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Version: Version of Record

Link(s) to article on publisher's website:

<http://dx.doi.org/doi:10.21954/ou.ro.00010088>

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Antibiotics in Mycobacteria-Macrophage System and its
Consequences at both Microorganism and Host Cell Level

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A thesis submitted in Fulfillment of the Requirements of the Open
University (U.K.) for the Degree of Doctor of Philosophy

Life Sciences

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(ICGEB), Trieste, Italy.

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May 2010

Date of Submission: 6 August 2009
Date of Award: 2 July 2010

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ACKNOWLEDGMENTS

I would like to express my deep and sincere gratitude to my supervisor, Dr. Rodolfo Garcia for having given me the opportunity of coming to the International Centre for Genetic Engineering and Biotechnology (ICGEB) and working on an exciting research project that culminated in this Thesis, Rodolfo, I truly appreciated the amount of time and energy that you invested in guiding me throughout this work, your support in the most critical moments, the many enriching scientific discussions.

I am deeply grateful to Professor Francisco Baralle for his essential support.

I am profoundly grateful to my external supervisor Prof. Siamon Gordon, Sir William Dunn School of Pathology, Oxford University, for his constructive comments and suggestions during this work.

I wish thank Dr. Eulalia Ximenes, from Department of Antibiotics, Federal University of Pernambuco, Recife, Brazil, Prof. Roberto Luzzati, MD, Ospedale Maggiore di Trieste, Trieste, Italy, Dr. Elena Banfi, from Università degli Studi di Trieste, Department of Biomedical Sciences, Trieste, Italy, and Avidin Ltd., Szeged, Hungary for kindly provide the compounds used in this work.

I wish to express my warm and sincere thanks to Li-Jun Tang for the sincere friendship and for the many helps in the beginning of this work, Marco Bestagno and Julian Pulecio for the guidance in my first steps into FACS, Paola Massimi for the instructions in the fluorescent microscope, and Cristiana Stuanì for many helps.

I owe my loving thanks to my beloved wife Janaina Oliveira, and our little daughter Martina. I really appreciated her sacrifice to stay together with me abroad. Without her encouragement and understanding would have been impossible for me to finish this work. My special gratitude to my family, especially to my parents, for their support and encouragement during this four year away from home.

TABLE OF CONTENTS

List of Figures	10
List of Tables	16
Summary	17
Abbreviations	19
Rationale and Aims	25
 INTRODUCTION	 26
1. The genus <i>Mycobacterium</i>	26
2. The Mycobacterial cell wall	28
3. Dormancy	31
4. Strategies to control mycobacterial infection	31
4.1. Chemotherapy	31
4.1.1. Treatment of non-tuberculous disease	32
4.1.2. Treatment of tuberculous disease	34
4.1.3. Approaches to develop new anti-mycobacterial compounds	35
4.2. Vaccination	37
4.2.1. Bacillus Calmatte-Guérin (BCG)	37
4.2.2. New vaccines against TB	38
5. The immune response against mycobacterial infections	39
5.1. Phagocytes / Antigen presenting cells	39
5.1.1. Neutrophils	40
5.1.2. Dendritic cells (DCs)	41
5.1.3. Macrophages	42
5.2. Phagocytosis and the phagosome	44

5.3. Macrophage activation	47
5.3.1. Classical activation of macrophage	47
5.3.1.1. IFN- γ signalling pathway	48
5.3.1.2. Toll-Like Receptors	50
5.3.2. Alternative activation of macrophages	52
5.3.3. Regulatory (Reg) macrophages	53
5.4. The use of THP-1 cells to study mycobacterium-macrophage interactions	53
5.5. Killing mechanisms of macrophages	55
5.5.1. Superoxide/ROS formation and degradation	56
5.5.1.1. NADPH oxidase	56
5.5.1.2. Superoxide dismutases	57
5.5.2. Nitric oxide generation	58
6. Binding receptors involved in macrophage recruitment, signalling and internalisation.	60
6.1. Complement Receptor 3	61
6.2. Mannose Receptor	62
6.3. CD14	62
6.4. Scavenger receptors	63
6.5. Fc γ receptors (Fc γ Rs)	64
7. Antigen presentation	65
7.1. Molecules responsible for the immunological synapse on the APC	66
7.1.1. Major Histocompatibility class I molecules (MHCI)	66
7.1.2. Major Histocompatibility class II molecules (MHCII)	67
7.1.3. Co-stimulatory molecules (B-7 family, CD83, ICAM-1)	68

7.1.3.1. B7 family	68
7.1.3.2. CD83	69
7.1.3.3. ICAM-1 (CD54)	69
8. Cytokine secretion	70
9. Mycobacterial infection of macrophages	73
9.1. Mycobacteria and TLRs	73
9.2. Subversion of the immune response by mycobacteria	74
9.2.1. Defences against oxidative mechanisms	74
9.2.2. Effect of mycobacteria on phagosome maturation	75
9.2.3. Other effects of mycobacteria on macrophage function	76
10. Potential application of natural compounds in human disease	77
10.1. Quinones	79
10.2. Other compounds causing microbial growth arrest used during the present work	84
11. Rationale and strategy of the present work	84
 MATERIALS AND METHODS	 86
1. Compounds of potential antibiotic activity	86
2. Main solutions	86
3. Cell cultures	87
3.1. Mammalian cell cultures	87
3.2. <i>Mycobacterium avium</i> cultures	87
3.2.1. Growth on agar	88
3.2.2. Growth in liquid medium	88
3.2.3. Susceptibility of <i>M. avium</i> to potentially antibiotic compounds (growth in liquid culture)	88

3.2.4. Bacteriostasis against bacterial killing	89
3.2.5. Intracellular proliferation of <i>M. avium</i>	89
3.2.5.1. Intracellular <i>M. avium</i> growth arrest by test compounds	90
3.3. <i>Staphylococcus aureus</i> and <i>Salmonella typhimurium</i> cultures	90
3.3.1. Susceptibility of <i>S. aureus</i> and <i>S. typhimurium</i> to test compounds (growth in liquid culture)	90
4. Cell morphology	91
5. Apoptosis and necrosis	91
6. Binding and internalisation of particles and bacteria	92
6.1. Latex beads	92
6.2. <i>S.aureus</i>	92
6.3. <i>S. typhimurium</i>	92
6.4. <i>M. avium</i>	93
6.4.1. Preparation of <i>M. avium</i> -FITC	93
6.4.1.1. Binding and internalisation by flow Cytometry	93
6.4.1.2. Binding and internalisation by fluorescent microscopy	93
7. Cell surface markers	94
8. Cytokine secretion	95
9. Subcellular fractionation	96
10. Protein Electrophoresis and Immunoblotting	96
11. Proteomics	97
11.1. Protein isolation and solubilisation	97
11.2. Bidimensional separations (IEF/SDS-PAGE)	97
11.3. Gel staining. Calculation of pI/s and molecular masses	98
11.4. Protein digestion	98
11.5. MALDI-TOF/TOF analysis and database searching	99

12. Statistical analysis	100
RESULTS	101
Chapter 1. Anti-mycobacterial activity and drug cytotoxicity	101
1. Anti-mycobacterial activity (mycobacteria in liquid culture)	103
2. Cytotoxicity toward THP-1 macrophages: Cell morphology, apoptosis and necrosis	105
3. Intracellular anti-mycobacterial activity	107
4. Distinguishing between bactericidal or bacteriostatic activity of lapachol	109
Chapter 2. Macrophage functions in response to activation	115
1. Endoplasmic reticulum (ER) stress	120
2. Oxygen metabolism	120
3. Oxidative stress	124
4. L-arginine metabolism	124
5. Expression of pattern recognition, phagocytic and adhesion receptors	126
5.1. Expression of TLR2	126
5.2. Expression of CD14	127
5.3. Expression of CD18	130
5.4. Expression of Fc receptors	130
5.5 Expression of Mannose Receptor (MR)	135
5.6 Expression of Scavenger Receptor AI (SR-AI)	135
5.7. Expression of CD87 (uPAR)	138
6. Phagocytosis	138
6.1. Latex beads	140
6.2. <i>S. aureus</i>	140
6.3. <i>S. typhimurium</i>	140
6.4. <i>M. avium</i>	141

7. Secretion of pro- and anti-inflammatory cytokines	143
7.1. TNF- α	144
7.2. IL-1 β	145
7.3. IL-10	145
 Chapter 3. IFN- γ responsiveness and antigen presentation	154
1. Expression of IFN- γ R1	156
2. Expression of CD64 (Fc γ RI)	158
3. Expression of CD54 (ICAM-1)	158
4. Expression of receptors involved in antigen presentation	158
4.1. Major histocompatibility molecule II (MHCII)	158
4.2. Expression of co-stimulatory receptors	161
 Chapter 4. Proteomics approach to the effect of lapachol on macrophage activation.	166
1. Changes induced by lapachol in resting cells	169
2. Influence of lapachol on IFN- γ -regulated protein expression levels	171
2.1. Protein folding	177
2.2. Glycolysis	178
2.3. Post-translational modifications	179
2.4. Proteolysis	180
2.5. Actin-binding	181
3. Influence of lapachol on P3CSK4-regulated protein expression levels	182
3.1. Actin-binding	188
3.2. Metabolism	190
3.3. Proteolysis	192
3.4. ER stress	193

3.5. Redox state	193
GENERAL DISCUSSION	196
REFERENCES	203

LIST OF FIGURES

Figure 1 : Mycobacterial cell wall structure.	29
Figure 2 : Process of phagosome formation and maturation.	46
Figure 3 : IFN- γ signalling pathway.	49
Figure 4 : TLR2 signalling pathway.	51
Figure 5 : NADPH oxidase complex assembly and superoxide generation.	57
Figure 6 : Superoxide dismutase and peroxidase reactions (protection against oxidative stress).	58
Figure 7 : L-arginine metabolism.	59
Figure 8 : Peroxynitrite anion generation.	60
Figure 9 : Chemical structure of 1,4-naphthoquinones.	80
Figure 10 : Naphthoquinone reduction by NADPH-cytochrome P450 reductase	81
Figure 11: Chemical structure of 9A and F2 compounds used in this study.	84
Figure 12: Effect of putative anti-mycobacterial compounds on THP-1 macrophage morphology.	106
Figure 13 : Lapachol and AC201 do not induce necrosis or apoptosis of THP-1 macrophages	108
Figure 14 : Anti- <i>M. avium</i> activity of lapachol (A) , AC201 (B), and three first line antibiotics (C) against intracellular <i>M. avium</i> , within THP-1 macrophage-like cells	110
Figure 15 : Bacteriostatic effect of lapachol toward <i>M. avium</i>	111

Figure 16 : Experimental scheme of THP-1 differentiation and subsequent treatments of THP-1 macrophages.	116
Figure 17 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or AC201 on the endoplasmic reticulum (ER) stress of THP-1 macrophages.	121
Figure 18 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or AC201 on the expression of key proteins of oxygen metabolism by THP-1 macrophages	123
Figure 19 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or AC201 on MnSOD and CuZnSOD expression by THP-1 macrophages	125
Figure 20 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol on L-arginine metabolism by THP-1 macrophages.	126
Figure 21 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface TLR2 by THP-1 macrophages	128
Figure 22 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface CD14 by THP-1 macrophages	129
Figure 23 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface CD18 by THP-1 macrophages	131

Figure 24 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface CD64 by THP-1 macrophages	132
Figure 25 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface CD32 by THP-1 macrophages	133
Figure 26 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface CD16 by THP-1 macrophages	134
Figure 27 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface mannose receptor (MR) by THP-1 macrophages	136
Figure 28 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface Scavenger Receptor-A (SR-A) by THP-1 macrophages	137
Figure 29 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of CD87 by THP-1 macrophages.	139
Figure 30 : Influence of TLR2 agonism and lapachol on the binding and phagocytosis of latex beads, <i>M. avium</i> , <i>S. aureus</i> and <i>S. typhimurium</i>	142
Figure 31 : Phagocytosis of <i>M. avium</i> by resting, activated and/or lapachol-treated THP-1 macrophages, evaluated by fluorescence microscopy.	143

Figure 32 : Cytokine secretion by resting, lapachol treated, TLR2-agonised, IFN- γ -treated and <i>M. avium</i> -infected THP-1 macrophages	146
Figure 33 : Effect of TLR2-agonism and lapachol treatment on the expression of surface IFN- γ R1 by THP-1 macrophages.	157
Figure 34 : Effect of TLR2 agonism and lapachol treatment on the total (surface + cytoplasmic) expression of IFN- γ R1 by THP-1 macrophages	159
Figure 35 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol on the surface expression of MHCII and ICAM-1 (CD54) by THP-1 macrophages	160
Figure 36 : Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface CD80 by THP-1 macrophages	161
Figure 37: Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface CD83 by THP-1 macrophages	162
Figure 38: Effect of activation, <i>M. avium</i> infection and treatment with lapachol or traditional antibiotics on the expression of surface CD86 by THP-1 macrophages	163
Figure 39: Representative cytosolic (A) and membrane (B) protein maps (2D-PAGE) of THP-1 macrophages untreated (Control) or treated with lapachol.	170

Figure 40 : Representative cytosolic protein maps (2D-PAGE) of	
THP-1 macrophages untreated (A) or treated with lapachol (B),	
IFN- γ (C) or IFN- γ + lapachol (D)	172
Figure 41 : Representative membrane protein maps (2D-PAGE) of	
THP-1 macrophages untreated (A) or treated with lapachol (B),	
IFN- γ (C) or IFN- γ + lapachol (D)	173
Figure 42 : Detail of the alterations induced by lapachol on IFN- γ -regulated	
cytosolic protein expression	175
Figure 43 : Detail of the alterations induced by lapachol on IFN- γ -regulated	
membrane protein expression	176
Figure 44 : Functional classification of the IFN- γ -regulated proteins the	
expression of which was influenced by lapachol	177
Figure 45 : Representative cytosolic protein maps (2D-PAGE) of	
THP-1 macrophages untreated (A) or treated with lapachol (B),	
P ₃ CSK ₄ (C) or P ₃ CSK ₄ + lapachol (D)	183
Figure 46 : Representative membrane protein maps (2D-PAGE) of	
THP-1 macrophages untreated (A) or treated with lapachol (B),	
P ₃ CSK ₄ (C) or P ₃ CSK ₄ + lapachol (D)	184
Figure 47 : Detail of the alterations induced by lapachol on P ₃ CSK ₄ -regulated	
cytosolic protein expression	186
Figure 48 : Detail of the alterations induced by lapachol on P ₃ CSK ₄ -regulated	
membrane protein expression	187

Figure 49: Functional classification of the P_3CSK_4 -regulated proteins the expression of which was influenced by lapachol	188
Figure 50: Detail of two of the alterations induced by lapachol on the cytosolic protein expression of resting cells	190

LIST OF TABLES

Table 1: Main characteristics of the drugs currently used in anti-tubercular treatments.	33
Table 2: Susceptibility of <i>M. avium</i> , <i>S. aureus</i> , and <i>S. typhimurium</i> to putative antibiotics.	104
Table 3: Proteins differentially expressed upon IFN- γ -treatment whose expression levels were affected by lapachol.	174
Table 4: Proteins differentially expressed upon P ₃ CSK ₄ -treatment whose expression levels were affected by lapachol.	185

SUMMARY

To select potentially therapeutic anti-mycobacterial agents, 9 compounds were screened on the basis of their capacity to arrest *M. avium* growth in liquid broth. Out of the 8 compounds that proved suitable, 2 were singled out upon evaluation of their apoptotic/necrotic effects on THP-1 macrophage-like cells. One of them controlled poorly *M. avium* intra-macrophage growth and showed deleterious effects on some function parameters of resting and activated host cells that led to its elimination as a potential antibiotic. The remaining compound was the substituted hydroxy-naphthoquinone lapachol, a natural product from a South American tree bark.

Lapachol was bacteriostatic toward *M. avium*, did not induce apoptosis or necrosis of THP-1 macrophages at $\leq 32 \mu\text{g/mL}$ ($132 \mu\text{M}$) and arrested the intra-macrophage growth of *M. avium* at the lower dose of $16 \mu\text{g/mL}$ ($66 \mu\text{M}$). The study of the effects of lapachol on the expression of function parameters and cytokine secretion by THP-1 macrophages showed that it immunomodulated resting and TLR2-agonised human macrophage functions, some of which can lead to improved host cell responses to infection. Favourable effects were increases in IFN γ R1 and MHCII surface expression and a marked inhibition of IL-10 secretion. The up-regulation of IFN γ R1 and MHCII can potentially improve the capacity to respond to IFN γ and present antigens, respectively. The inhibition of IL-10 secretion would prevent macrophage de-activation, improving the capacity of macrophages to control the intracellular growth of *M. avium*. Lapachol did not affect important aspects of host cell function such as ER or oxidative stress and TLR2 agonism-induced capacity to produce oxygen and nitrogen metabolites and secrete TNF- α . The drug resulted in a reduction of IL-1 β and TNF- α secretion only from resting and IFN- γ -treated cells, and in a decrease in latex bead and *M. avium* internalization. A proteomic approach to study the influence of lapachol on the expression of proteins modulated by IFN- γ or TLR2 agonism showed important reductions in the expression levels of three proteases, the ER protein

DnaJ homolog that forms part of complexes together with grp78 regulates grp78 activity and two cytoskeletal proteins, L-plastin and fascin. The down-regulation of these cytoskeletal proteins could hypothetically affect cell migration and phagocytosis. Lapachol treatment resulted in important increases in cytosolic protein disulfide isomerase A3 (ERp57), thought to be involved in Stat3 complexing and in disulfide bond cleavages coupled to protein degradation, and glucose-6-P-dehydrogenase, involved in NADPH generation and, as a consequence, in the protection against damage by hydrogen peroxide. The results obtained during this work suggest the convenience of *in vivo* experiments to further test lapachol regarding its potential as a new anti-mycobacterial agent and immunomodulator.

ABBREVIATIONS

AaMΦs:	Alternatively activated macrophages
ADC:	Albumin-dextrose complex
ADCC:	Antibody-dependent cell-mediated cytotoxicity
ANOVA:	Analysis of variance
APC:	Antigen presenting cells
AraLAM:	Uncapped lipoarabinomannan
ATP:	Adenosine-5'-triphosphate
BCG:	Bacillus Calmatte-Guérin
BCIP:	5-Bromo-4-chloro-3-indolyl phosphate
BIP:	Binding immunoglobulin protein
Ca ⁺² :	Calcium
CaMΦs:	Classically activated macrophages
CAP1:	Adenylyl cyclase-associated protein 1
Cat:	Cathepsin
CD:	Cluster of differentiation
CF:	Cord factor
CFP:	Culture filtrate protein
CFU:	Colony forming unit
CNS:	Central nervous system
CR3:	Complement receptor 3
CSF-1:	Colony-stimulating-factor-1
CTLA-4:	Cytotoxic T-lymphocyte antigen-4
Cu:	Cooper
DC:	Dendritic cell

DC-SIGN:	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
EEA-1:	Early endosome antigen-1
ELISA:	Enzyme-linked Immunosorbent Assays
ER:	Endoplasmic reticulum
FACS:	Fluorescence-activated cell sorting
FcR:	Fc receptor
FCS:	Fetal calf serum
Fe ⁺³ :	Iron
FITC:	Fluorescein isothiocyanate
G6PD:	Glucose-6-phosphate 1-dehydrogenase
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GAS:	Gamma-activated sequence
GFP:	Green fluorescent protein
GI:	Gastrointestinal
GM-CSF:	Granulocyte-monocyte colony stimulating factor
Grp78:	Glucose regulated protein
H ₂ O ₂ :	Hydrogen peroxide
HCl:	Hydrochloric acid
HIV:	Human immunodeficiency virus
HPLC:	High pressure liquid chromatography
Hsp90:	Heat shock protein 90
ICAM:	Inter-Cellular Adhesion Molecule

IEF:	Isoelectrofocusing
IFN- γ :	Interferon-gamma
IFN- γ R1:	Interferon-gamma receptor 1
Ig:	Immunoglobulin
IL:	Interleukine
IPG:	Immobilized pH-gradient
IRAK 1:	Interleukin-1 receptor-associated kinase 1
Jak:	Janus kinase
LAM:	Lipoarabinomannan
LAMP:	Lysosome-associated membrane protein
LB:	Luria agar
LDL:	Low density lipoproteins
LFA-1:	Lymphocyte function-associated antigen-1
LM:	Lipomannan
LPS:	Lipopolysaccharide
LTA:	Lipoteichoic acid
<i>M. abscessum</i> :	<i>Mycobacterium abscessum</i>
<i>M. avium</i> :	<i>Mycobacterium avium</i>
<i>M. bovis</i> :	<i>Mycobacterium bovis</i>
<i>M. chelonae</i> :	<i>Mycobacterium chelonae</i>
<i>M. leprea</i> :	<i>Mycobacterium leprea</i>
<i>M. marinum</i> :	<i>Mycobacterium marinum</i>
<i>M. smegmatis</i> :	<i>Mycobacterium smegmatis</i>
<i>M. tuberculosis</i> :	<i>Mycobacterium tuberculosis</i>
<i>M. ulcerans</i> :	<i>Mycobacterium ulcerans</i>
M-CSF:	Macrophage colony-stimulating factor
MABA:	Microplate alamar blue assay

MAC:	<i>M. avium</i> complex
Mac-1:	Macrophage antigen-1
mAGP:	Mycolyl arabinogalactan-peptidoglycan
ManLAM:	Lipoarabinomannan capped with mannose
MARCO:	Macrophage receptor with collagenous structure
MCP-1:	Monocyte chemoattractant protein-1
MDR:	Multidrug resistance
MFI:	Median fluorescence intensity
MHC:	Major Histocompatibility Complex
MIC:	Minimal Inhibitory Concentration
ManR:	Mannose Receptor
Mn:	Manganese
MS:	Mass spectrometry
MSR-1:	Macrophage scavenger receptor-1
MyD88:	Myeloid differentiation primary response gene (88)
NADPH:	Nicotinamide adenine dinucleotide phosphate
NaN ₃ :	Sodium azide
NaOH:	Sodium hydroxide
NBT:	Nitro blue tetrazolium chloride
NF-κB:	Nuclear factor-kappa B
NK:	Natural killer
NO:	Nitric oxide
NOS2:	Nitric oxide synthase 2
O ₂ ⁻ :	Superoxide
OADC:	Oleic acid-albumin-dextrose complex
P ₃ CSK ₄ :	<i>N</i> -palmitoyl- <i>S</i> -[2,3-bis(palmitoyloxy)-(2 <i>R</i> , <i>S</i>)-propyl]-(<i>R</i>)-cysteinyl-seryl-(lysyl) ₃ -lysine

PAMPs:	Pathogen-associated molecular patterns
PBS:	Phosphate buffered saline
PDI:	Protein disulfide-isomerase
PE:	Phycoerythrin
PFA:	Paraformaldehyde
pH:	Potential for hydrogen ion concentration
PI3-K:	Phosphatidyl-inositol 3-kinase
PI3P:	Phosphatidyl-inositol-3-phosphate
pI:	Isoelectric point
PK:	Pyruvate kinase
PILAM:	phosphoinositide-capped LAM
PIM:	Phosphatidylinositol mannosides
PMA:	Phorbol 12-myristate 13-acetate
PRRs:	Pattern recognition receptors
Reg:	Regulatory
RegMΦs:	Regulatory macrophages
RNA:	Ribonucleic acid
RNI:	Reactive nitrogen intermediate
ROS:	Reactive oxygen species
RPMI:	Roswell Park Memorial Institute medium
RT:	Room temperature
<i>S. aureus</i> :	<i>Staphylococcus aureus</i>
SDS:	Sodium Dodecyl Sulphate
SDS-PAGE:	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
S:E:	Standard error
SMAC:	Supramolecular activation cluster
SOCS:	Suppressors of cytokine signaling

SOD:	Superoxide Dismutase
SR-AI:	Scavenger receptor-AI
ssGPL:	Serovar-specific glycopeptidolipids
STAT:	Signal transducers and activator of transcription
<i>S. typhimurium: Salmonella typhimurium</i>	
SUMO:	Small ubiquitin-like modifier
TACO:	Tryptophan/aspartate-containing coat protein
TAP:	Transported associated with antigen processing
TB:	Tuberculosis
TCA:	Trichloroacetic acid
TCR:	T cell receptor
TGF- β :	Transforming growth factor-beta
TLR:	Toll-like receptor
TNF- α :	Tumor necrosis factor-alpha
TRAF6:	Tumour necrosis factor-receptor associated factor 6
uPAR:	Urokinase-type plasminogen receptor
UPR:	Unfolded protein response
VPS34:	Phosphatidyl-inositol-3-kinase
WHO:	World Health Organization
XDR:	Extensively drug-resistant
Zn:	Zinc

RATIONALE AND AIMS

M. avium is a non-tuberculous, facultative intracellular pathogen that shares a number of characteristics with *M. tuberculosis* concerning intra-macrophage growth and inhibition of host cell responses. Besides, *M. avium* is an agent increasingly causing disease worldwide. New compounds, synthesized or isolated from natural sources, are not often tested for their efficacy against microorganisms growing intracellularly, as is the case for pathogenic mycobacteria. Even fewer candidate compounds are studied in relationship with their effects on mammalian host cells. This is an important issue concerning mycobacterial infections, since pathogen growth control or elimination requires treatments with antibiotics for up to 2 years in the case of resistant mycobacterial strains or in non-tuberculous disease, like the one caused by *M. avium*.

The aim of this thesis was to test new compounds for their anti-mycobacterial activity, using *M. avium* as the target bacterium, and for their effect on resting, activated and *M. avium*-infected host cells (THP-1 macrophages). By establishing criteria for the *in vitro* selection of putative anti-mycobacterial drugs, they can be shortlisted before moving to *in vivo* studies. The screening of candidate compounds was based on their effects on parameters indicative of functions such as viability, oxygen and nitrogen metabolism, endoplasmic reticulum stress, migration, phagocytosis, antigen presentation and cytokine secretion. A proteomic approach was finally used for the evaluation of the effects of the naphthoquinone lapachol, which was the only compound left after the screening mentioned above.

INTRODUCTION

1. The genus *Mycobacterium*

The genus *Mycobacterium* is a member of a GC rich group of Gram-positive bacteria consisting mainly of non-pathogenic, environmental organisms. They belong to the Actinobacteria genera and are closely related to *Streptomyces*, which are characterized by forming spores. However, the ability of *Mycobacterium* to form spores is questioned. Ghosh et al (Ghosh, Larsson et al. 2009) demonstrated the presence of spores in old cultures of *Mycobacterium marinum*, but Traag et al (Traag, Driks et al. 2010) failed to detect them. Moreover, the latter reported the absence of orthologs of conserved genes responsible for endospore formation in high GC mycobacteria.

Despite the fact that most of the species within the *Mycobacterium* genus are environmental, some like *M. tuberculosis*, *M. marinum*, *M. ulcerans*, *M. leprea*, and *M. avium* complex developed the ability to reside and proliferate within mammalian host cells and this made them successful pathogens. Mutant strains of *M. tuberculosis* that do not express the pathogenicity-related genes *sigE* or *erp* fail to grow within macrophages in culture and show a reduced pathogenicity *in vivo* (Berthet, Lagranderie et al. 1998; Manganelli, Voskuil et al. 2001; Ando, Yoshimatsu et al. 2003). SigE is a sigma factor known to be involved in responses to heat shock, oxidative and low pH stress. Erp encodes exported repetitive proteins that may be involved in phagosome maturation control. Thus, it can be concluded that replication within macrophages is an important requirement for mycobacterial pathogenicity. It is conceptually interesting to try and understand why a pathogen that can survive and replicate on its own did not avoid phagocytosis, the cells meant to kill them, but instead developed mechanisms to gain access and survive in the intracellular environment of those cells.

Mycobacterium tuberculosis is an efficient, successful human pathogen causing tuberculous disease. It infects one third of the world population, and out of more than 9

million new cases per year almost 15% are HIV positive patients. In spite of being a curable disease in most cases, tuberculosis still results in more than 2 million deaths per year (WHO 2009). *Mycobacterium avium* complex, *Mycobacterium abscessus* and other non-tuberculous organisms cause infections that had been previously associated only with immune deficiencies such as AIDS and cystic fibrosis but have now extended among the population at large. These organisms are ubiquitous in the environment and particularly in acid soils and hypo-oxygenated, organic matter-rich water (Griffith, Aksamit et al. 2007; Piersimoni and Scarparo 2008).

After being internalized by phagocytes into a cytoplasmic, membrane-bound phagosome, pathogenic mycobacteria inhibit cellular responses controlling infection. Among these responses, mycobacteria particularly target autophagy (Deretic, Singh et al. 2006). Kumar et al (Kumar, Nath et al. 2010) have recently reported a genome analysis of macrophages infected by different strains of *M. tuberculosis* and concluded that autophagy inhibition is a general strategy employed by pathogenic mycobacteria to subvert the host immune response, in association with the induction of an enhancement in the expression of anti-inflammatory molecules. Moreover, mycobacteria also block phagosome acidification by exclusion of ATPase pumps (Pieters 2001; Huynh and Grinstein 2007) and prevent the fusion of the phagosomes with lysosomes (Via, Deretic et al. 1997). These alterations of the host cell behaviour create a niche that favours mycobacterial survival and replication. Pathogenic mycobacteria also inhibit apoptosis and instead induce necrosis, which favours bacterial spreading in the host organism.

During the establishment of infections, the replication of pathogenic mycobacteria has a special requirement for iron storage, which is an important co-factor of certain enzymes such as KatG, a catalase/oxidase. This is a protective enzyme that needs iron to be able to scavenge hydrogen peroxide. In consequence, iron deficiency can generate bacterial oxidative stress (Dussurget and Smith 1998). Macrophages are an important source of iron

since they can phagocytose senescent erythrocytes. However, the availability of free iron at phagosomal pH (~6.1-6.5) is low, due to its poor solubility. In order to obtain the required amount of iron, mycobacteria have developed mechanisms to compete with the host for the scarce amount available. Mycobacteria are capable of synthesizing siderophores that compete and remove iron from the human iron-storing proteins transferrin and ferritin, which are siderophores themselves. Two types of mycobacterial siderophores have been described, the cell wall anchored mycobactin and extracellular carboxymycobactin, secreted by pathogenic microorganisms, and exochelin, secreted by saprophytes. These siderophores provide mycobacteria with the necessary amount of iron for their metabolism, therefore they constitute an important target for new anti-mycobacterial drugs. For instance, the anti-mycobacterial drug *p*-aminosalicylic acid is speculated to exert its activity by inhibiting mycobactin rather than folic acid synthesis (Ratledge 2004; Boelaert, Vandecasteele et al. 2007).

2. The mycobacterial cell wall

Mycobacteria are slender, aerobic restricted and facultative intracellular rods that infect most usually mononuclear phagocytes (Jozefowski, Sobota et al. 2008). Their lipid-rich cell wall consists of complex lipids containing long chain fatty acids and mycolic acids, waxes, glycolipids, proteins, polysaccharides, and lipoglycans. It is responsible for an intrinsic resistance to antimicrobial agents as well as playing an important role in intracellular survival (Nigou, Gilleron et al. 2003; Primm, Lucero et al. 2004).

The mycobacterial cell wall is composed by an upper and a lower section. The lower section consists of an asymmetric bilayer membrane. The most common phospholipids found in mycobacterial membrane are phosphatidylglycerol, diphosphatidylglycerol, phosphatidyl-ethanolamine and phosphatidylinositol mannosides (PIMs). Beyond the membrane there is a cell wall core composed of peptidoglycan, arabinogalactan (AG) and mycolic acid, also called mycolyl-arabinogalactan-peptidoglycan (mAGP), where the

peptidoglycan is covalently attached via a phosphodiester bond to the complex arabinogalactan-mycolic acid. This core is essential for cell viability and understanding its biosynthesis is fundamental for the development of new anti-mycobacterial drugs. The upper section of the cell wall is composed of free lipids such as phthiocerol dimycoserolate, cord factor (CF) (in *M. tuberculosis*) and sulfolipids. Embedded in the cell wall there are PIMs, lipomannan (LM) and lipoarabinomannan (LAM), which play an important role in mycobacterial virulence (Figure 1) (Brennan and Nikaido 1995; Chatterjee 1997; Brennan 2003).

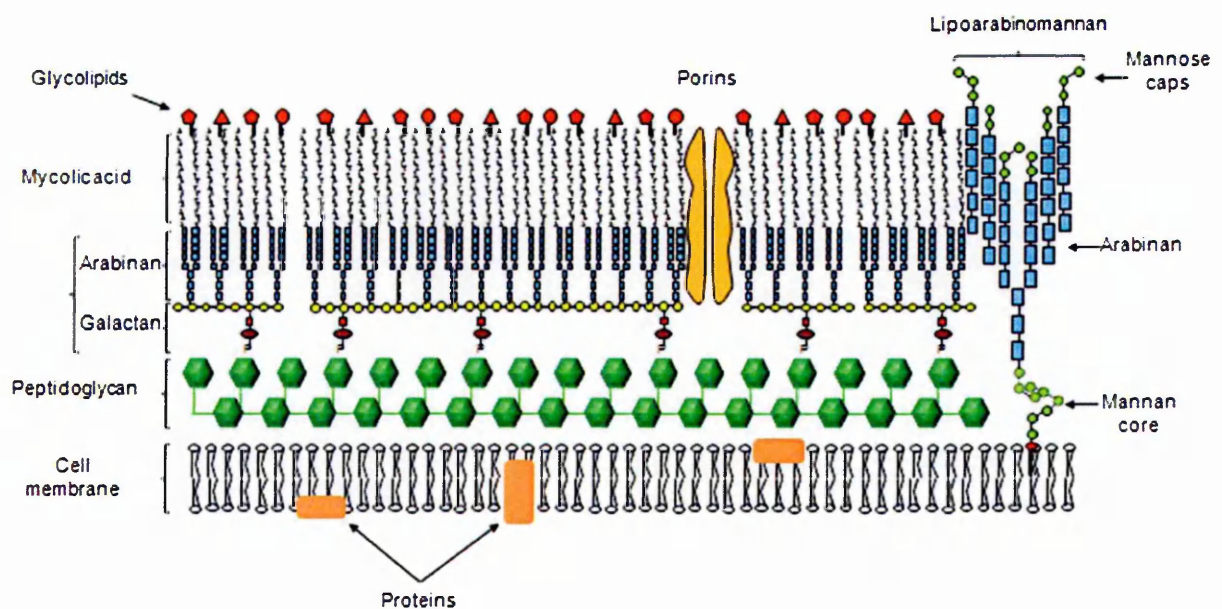


Figure 1. Mycobacterial cell wall structure. Above the cell membrane there is the core of mycobacterial cell wall, consisting of peptidoglycan covalently attached to arabinogalactan and mycolic acids (mycolyl-AG-peptidoglycan complex). In non-tuberculous mycobacteria the core is covered by a layer composed of different free glycolipids, which confer virulence. This figure has been extracted with modifications from Chatterjee, D. (Chatterjee and Khoo 2001).

Most mycobacterial virulence factors are cell wall glycolipids. Lipoglycans are recognized by cells from the immune system as pathogen-associated molecular patterns (PAMPs), a phenomenon that constitutes the first stage of induction of immune responses. Phosphatidyl-inositol mannosides and their hyperglycosylated derivatives, lipomannan (LM) and lipoarabinomannan (LAM) are the most important glycolipids present in the mycobacterial cell wall. LAM and LM are expressed in slow-growing, pathogenic mycobacteria, are non-covalently anchored to the plasma membrane and extend to the external part of the cell wall (Briken, Porcelli et al. 2004; Crellin, Kovacevic et al. 2008). In virulent strains, LAMs are capped with mannose generating a molecule called ManLAM. Fast-growing, atypical mycobacteria express phosphoinositide-capped LAM (PILAM). ManLAM is known to be a potent inhibitor of phagosomal maturation (Fratti, Chua et al. 2003). This occurs following ManLAM incorporation into macrophage membrane rafts (Welin, Winberg et al. 2008). Uncapped LAM (AraLAM) is the glycolipid present in the avirulent mycobacteria *M. smegmatis* and *M. chelonae* (Nigou, Gilleron et al. 2003). ManLAM is recognized by the mannose receptor (ManR) of macrophages, which promotes phagocytosis and induces in general an anti-inflammatory response, whilst LM, AraLAM and PILAM trigger the TLR2 signalling pathway and induce macrophage activation (Wieland, Knapp et al. 2004; Jozefowski, Sobota et al. 2008).

The *M. avium* subsp. *avium* cell wall is a complex array of hydrocarbon chains containing the arabinogalactan-peptidoglycan-mycolic acid core found in all mycobacteria, surrounded by a second electron-dense layer made up in part of serovar-specific glycopeptidolipids (ssGPL) exclusive to *M. avium* complex. ssGPLs consist of non-specific GPLs common to many environmental mycobacteria, modified by the addition of serovar-specific oligosaccharide side chains (Chatterjee and Khoo 2001; Laurent, Hauge et al. 2003; Schorey and Sweet 2008).

3. Dormancy

A hallmark of mycobacterial behaviour is the capacity of the bacillus to go into a dormant state at low oxygen tensions. In order to survive, the metabolic machinery starts to use nitrate as the final electron acceptor. This situation was reproduced *in vitro* by Wayne and Hayes (Wayne and Hayes 1996). In these conditions bacteria do not replicate and show a limited metabolic activity, with scarce synthesis of new RNA and proteins, a strong induction of respiratory nitrate reductase activity, and a change in energy metabolism. This allows mycobacteria to survive in immunocompetent hosts for many years. In addition, this feature makes them non-susceptible to many antibiotics used to treat active infections (Honer zu Bentrop and Russell 2001; Wayne and Sohaskey 2001; Zahrt 2003; Sohaskey 2008). However, dormant bacteria have been reported to be sensitive to some drug associations (Iona, Giannoni et al. 2007; Filippini, Iona et al. 2010).

4. Strategies to control mycobacterial infection

4.1. Chemotherapy

An effective anti-mycobacterial compound must either kill bacteria or suppress their replication within the host cell (Amaral, Martins et al. 2007). The usual therapeutic schemes to treat mycobacterium infections employ combinations of bacteriostatic and bactericidal compounds, chosen according to their effectiveness and cost as first or second-line compounds (Table 1). First-line antibiotics are more effective, cheaper, and present less side effects than second –line compounds. These combinations are intended to minimize the risk of appearance of resistance. As opposed to bactericidal compounds that kill bacteria directly, bacteriostatic compounds prevent bacterial growth, therefore the host's immune system is needed to control the infection. However, this classification is not rigid and depends on factors such as the target organism (the same compound can be bactericidal or bacteriostatic toward different bacteria), the concentration and the tissue

penetration of the antibiotic drug. Since bacteriostatic compounds do not really eliminate the pathogen, bactericidal compounds are additionally necessary in some situations, e.g. when the infection foci are in tissues with poor blood penetration (bone) or in nervous tissue protected by a blood barrier, or when the host suffers from an immunological disorder, e.g. HIV (Pankey and Sabath 2004).

4.1.1. Treatment of non-tuberculous disease.

Disease caused by non-tuberculous mycobacteria requires long treatments, during which drug interactions, side-effects and patient compliance rates may be a problem. Ethambutol, rifampin, clarithromycin and azithromycin are widely prescribed for non-tuberculous disease therapy (Piersimoni and Scarparo 2008; Ballarino, Olivier et al. 2009). MAC infections are more difficult to cure than tuberculosis, and treatment includes the association of first and second line drugs for 10-12 months after sputum conversion. Among second-line compounds, fluoroquinolones have shown promising results (Jacobs 2004). Some drugs used for the treatment of other diseases have been found to be active against *M. avium* complex (MAC), such as the immunomodulators methotrexate and azathioprine, the anti-inflammatory 5-amino-salicylic acid and the immunosuppressants cyclosporine A, rapamycin and tacrolimus (Greenstein, Su et al. 2008; Juste, Elguezabal et al. 2008).

First Line Drugs				
Drug	Year Discovered	Mechanism of Action	Bactericidal / Bacteriostatic	Major Adverse Reactions
Isoniazid	1952	Cell wall synthesis inhibitor	Bactericidal	Hepatitis, peripheral neuropathy, lupus-like syndrome, drug interactions
Rifampin	1966	RNA synthesis inhibitor	Bactericidal	Drug interactions, orange color of body fluids, GI, hepatitis, fever, acute renal failure, hemolytic anemia
Pyrazinamide	1952	Disruption of electron transport across the membrane	Bacteriostatic	Hyperuricemia, gouty arthritis, rarely nephritis
Ethambutol	1961	Cell wall inhibitor	Bacteriostatic	Optic neuritis, exfoliative rash
Streptomycin	1944	Protein synthesis inhibitor	Bactericidal	Cochlear and vestibular toxicity, nephrotoxicity
Second Line Drugs				
Drug	Year Discovered	Mechanism of Action	Bactericidal / Bacteriostatic	Major Adverse Reactions
Capreomycin	1956	Protein synthesis inhibitor	Bactericidal	Cochlear and vestibular toxicity, nephrotoxicity
Kanamycin	1957	Protein synthesis inhibitor	Bactericidal	Cochlear and vestibular toxicity, nephrotoxicity
Amikacin	1974	Protein synthesis inhibitor	Bactericidal	Cochlear and vestibular toxicity, nephrotoxicity
Ethionamide	1956	Inhibitor of mycolic acid synthesis	Bactericidal	GI toxicity/hepatitis/dizziness
Aminosalicylic Acid (PAS)	1946	Inhibitor of folic acid	Bacteriostatic	GI toxicity, fever, rash
Cycloserine	1952	Inhibitor of peptidoglycan synthesis	Bacteriostatic	Dizziness, depression, CNS
Ciprofloxacin	1986	Inhibitor of DNA gyrase	Bactericidal	GI toxicity, CNS, tendon rupture
Ofloxacin	1995	Inhibitor of DNA gyrase	Bactericidal	GI toxicity, CNS, tendon rupture
Levofloxacin	1996	Inhibitor of DNA gyrase	Bactericidal	GI toxicity, CNS, tendon rupture
Moxifloxacin	1999	Inhibitor of DNA gyrase	Bactericidal	GI toxicity, CNS, tendon rupture
Gatifloxacin	1999	Inhibitor of DNA gyrase	Bactericidal	GI toxicity, CNS, tendon rupture
Clofazimine	1954	Binding of mycobacterial DNA and mRNA	Bacteriostatic	GI toxicity, cutaneous, ocular discoloration/pigmentation QT prolongation, dizziness

Table 1. Main characteristics of the drugs currently used in anti-tubercular treatments.

Information about first- and second-line antibiotics currently employed in tuberculosis treatments. Adapted from Laurenzi, M. et al (Laurenzi, Ginsberg et al. 2007), with modifications. GI: gastrointestinal; CNS : Central nervous system; QT : time between initial deflection of QRS complex to the end of T wave in electrocardiogram.

4.1.2. Treatment of tuberculous disease.

The chemotherapeutic scheme against *M. tuberculosis* infections recommended by the World Health Organization (WHO) consists in combinations of the five first-line drugs isoniazid, rifampin, pyrazinamide, streptomycin and ethambutol, in order to achieve good efficacy and tolerability. When strictly followed, this scheme results in a rate of cure exceeding 90% (Fattorini, Migliori et al. 2007). The association of drugs with different mechanisms of action is a general strategy to avoid the development of resistance and increase efficacy. However, the regime requires at least six months in the case of sensitive strains, which in itself is an important limitation. In fact, lack of patient adherence to the therapeutic schemes causes the appearance of resistant strains that can result in MultiDrug-Resistant (MDR) and eXtensively Drug-Resistant TB (XDR-TB). The long treatment needed for mycobacterial infections compared with those caused by other Gram-positive or negative bacteria is attributed to “physiological” heterogeneity of *M. tuberculosis*. Even when a bacterial population is genetically sensitive, a small proportion of the microorganisms can be phenotypically resistant and be present in a dormant or non-replicative state with a metabolic activity different from that of sensitive, actively replicating organisms. Co-infection with HIV represents another limitation for the treatment of mycobacterial infections due to drug interactions, apart from facilitating the progression from latent infection to active disease.

Bacteria develop resistance to antimicrobial compounds by mutation and selection of resistant individuals, in a mechanism termed vertical evolution, or by acquiring the genetic information that encodes resistance from other bacteria, called horizontal evolution (Tenover 2006). In the case of mycobacterium, development of resistance to first and second-line antibiotics seems associated with vertical evolution (Telenti, Imboden et al. 1993; Morris, Bai et al. 1995; Blanchard 1996). Mutations can confer resistance by altering the antibiotic target protein (Jugheli, Bzekalava et al. 2009), up-regulating antibiotic-inactivating enzymes as in the case of β -lactam (Kong, Schneper et al. 2010), changing a

pro-drug-activating enzyme (Slayden and Barry 2000), down-regulating or altering membrane proteins necessary for drug entry, or up-regulating the expression of efflux pumps (Ramon-Garcia, Martin et al. 2009).

MDR strains are resistant to isoniazid and rifampin, the two most effective first line drugs, and are responsible for 1-3 % of global TB cases. Treatment of MDR-TB is based on the bacterial susceptibility to the remaining first-line drugs, used in combination with second-line drugs (kanamycin, ofloxacin, capreomycin, ethionamide, *p*-aminosalicylic acid, and cycloserine). Treatments are longer and the cure rate is lower compared with TB due to sensitive strains. This increases the risk of appearance of further resistant strains (Di Perri and Bonora 2004; Chambers, Turner et al. 2005; Fattorini, Migliori et al. 2007).

The term eXtensively Drug-Resistant TB (XDR-TB) is used from 2006 for *M. tuberculosis* strains resistant to isoniazid and rifampin, to any fluoroquinolone and to at least one of the three second-line drug amikacin, capreomycin or kanamycin. These strains are associated with high mortality and their emergence is related to inadequate TB treatment and the HIV epidemic (Fattorini, Migliori et al. 2007; Elston and Thaker 2008; Jain and Mondal 2008; Madariaga, Lalloo et al. 2008). The appearance of XDR strains has led to the development of alternative treatments with the introduction of compounds usually employed in the treatment of other illnesses that proved to possess anti-mycobacterial activity (Amaral, Martins et al. 2008; Mor, Bingham et al. 2008; Kinnings, Liu et al. 2009).

4.1.3. Approaches to develop new anti-mycobacterial compounds

The reduced existing options of antimicrobial agents and the increase in resistant strains make necessary the development of new active compounds without cross-resistance. The aim of new compounds should be to reduce the length of treatment, which decreases the chances of new resistances, and to eliminate latent disease (Sensi 1989). A good

knowledge of mycobacterial biochemistry could result in discovering new specific targets for the fight against this microorganism. The sequencing of the *Mycobacterium tuberculosis* genome has provided valuable information about putative targets. Mdluli & Spigelman (Mdluli and Spigelman 2006) and Murphy, D.J. & Brown, J.R. (Murphy and Brown 2008) have reported several mycobacterial genes involved in key biochemical pathways such as cell wall and fatty acid biosynthesis. If these pathways are restricted to the microorganism, they would result in fewer or no side-effects for the host. Some of the gene products could also constitute possible molecular targets for dormant cells.

New active compounds are urgently needed for the treatment of mycobacterial infections, yet developing new drugs is time consuming. Label extension is an approach involving the extension of existing treatments for other diseases. This approach could shorten the times necessary to reach clinical use and reduce costs (Kinnings, Liu et al. 2009). For instance, immunomodulatory agents used to treat inflammatory bowel disease such as methotrexate, 5-amino-salicylic acid and azathioprine, and immunosuppressants such as cyclosporine A, rapamycin and tacrolimus have been recently reported to inhibit *M. avium subsp. paratuberculosis* growth (Greenstein, Su et al. 2008; Juste, Elguezabal et al. 2008). Similarly, the drugs entacapone and tolcapone, prescribed for Parkinson's disease, have been predicted to bind to the *M. tuberculosis* target enzyme InhA and could prove useful for the treatment of tuberculosis (Kinnings, Liu et al. 2009). Along the same lines, the neuroleptic compound thioridazine has been recently proposed for the treatment of MDR- and XDR-TB because it acts as an inhibitor of bacterial efflux pumps (Amaral, Martins et al. 2008).

High-throughput cell-free methods are another alternative to shorten screening times to test anti-mycobacterial compounds. The Alamar Blue assay (MABA) measures mycobacterial viability by enzymatic reduction of resazurin. Radiometric assays such as the BACTEC system have shortened the time to determine viability but employing radioactive isotopes is costly (Collins and Franzblau 1997). Arain has described a method

to quantify anti-mycobacterial activity based on luminescence, using strains carrying the luciferase gene (Arain, Resconi et al. 1996). However, all these methods deal with drug activity against mycobacteria grown in liquid broth. An anti-mycobacterial compound of therapeutic potential must be active against mycobacteria growing within host cells, usually macrophages. A high-throughput screening has been recently set up based on confocal microscopy images that can be used to study cell toxicity and for drug discovery (Zock 2009). The efficacy of this technique was demonstrated by screening the anti-plasmodial activities within red cells, employing fluorescent DNA staining of *Plasmodium falciparum* (Baniecki, Wirth et al. 2007). Using the same principle, Christophe et al infected mouse macrophages with GFP-expressing mycobacteria and screened the activity of candidate anti-mycobacterial compounds by measuring the fluorescence emitted by the intra-cellular mycobacteria (Christophe, Jackson et al. 2009). In this way they were able to demonstrate that nitrobenzamide had a good activity, which turned out to be a new mechanism of action due to the inhibition of arabinan biosynthesis.

4.2. Vaccination

4.2.1. Bacille Calmette-Guérin (BCG)

The vaccine against tuberculosis used at present was developed by Albert Calmette and Camille Guérin in 1921, using an attenuated virulent strain of *Mycobacterium bovis*. After 230 subcultures in potato-glycerine-ox bile for 11 years they produced an attenuated strain of *M. bovis* that was called bacille Calmette-Guérin (BCG) (Sakula 1983). This strain failed to cause tuberculosis in different animal models due to the loss or rearrangement of several genes that confer virulence (Behr, Wilson et al. 1999). BCG is the most widely used vaccine in the world. It has a good adjuvant activity and can elicit both humoral and cell-mediated immune responses. BCG is inexpensive compared with other live vaccines, and a single inoculum can give life-long protection. However, the efficacy of BCG ranges from excellent protection to no protection against TB. This wide

variation can be partly explained by the diversity of strains generated by the many passages performed in different laboratories, which have resulted in strains of different immunogenicity (Martin 2005). BCG can provide a good protection against TB in children. However, its low efficacy against pulmonary TB in adults fails to control the increase of new TB cases worldwide. BCG vaccination may be useful in preventing first infections but has no relevant effect on people already infected and in cases of disease reactivation (Agger and Andersen 2002).

4.2.2. New vaccines against TB

The increasing difficulty in controlling new TB cases is driving the development of vaccines either alternative or complementary to BCG. Several candidates based on living or non-living organisms are being tested, including some at the stage of pre-clinical trials (Girard, Fruth et al. 2005). *M. tuberculosis* attenuated strains are among the candidates to produce live TB vaccines. Their main advantage resides in the fact that many of the genes deleted from BCG during laboratory passages are still present in *M. tuberculosis*, such as ESAT-6 and CFP10, which have been reported to be important to promote protection against further *M. tuberculosis* challenge (Pym, Brodin et al. 2003). However, safety and stability problems must be solved concerning the use of live strains.

Because of the convenient attributes, safety, low cost, and easy mass production of BCG, a major effort is being made to obtain recombinant BCG strains expressing *M. tuberculosis* genes, thus creating a vaccine vehicle with increased protection efficiency against *M. tuberculosis* compared with wild type BCG (Ohara and Yamada 2001; Bastos, Borsuk et al. 2009). A study by Castañon-Arreola reported that vaccination with a recombinant BCG expressing a *M. tuberculosis* 38 kDa protein (rBCG38) increased the survival of mice after *M. tuberculosis* challenge, inducing a strong Th1 response (Castanon-Arreola, Lopez-Vidal et al. 2005). Grode et al (Grode, Seiler et al. 2005) described the use of a urease C-deficient rBCG expressing the membrane-perforating

protein listeriolysin of *Listeria monocytogenes*. The ingested organisms showed an acidic intraphagosomal pH that was optimum for listeriolysin activity, and this resulted in the formation of phagosomal membrane pores allowing antigen translocation into the cytoplasm followed by MHCI presentation to CD8⁺ T cells. The superior vaccine efficacy observed, as compared with parental BCG, is then based on improved cross-priming, which causes a stronger and more specific CD8⁺ T cell response.

Non-living vaccine candidates are subunit protein- or DNA-based. Subunit protein technology employs protein, lipid and/or carbohydrate components of *M. tuberculosis* known to be immunogenic as immunogenic particles. However, this strategy showed the same level of protection as BCG, is expensive and requires multiple boosters (Nor and Musa 2004). A more promising non-living vaccine employs plasmid DNA encoding antigens that can elicit protective response against tuberculosis. Gao et al (Gao, Yue et al. 2009) inserted sequences corresponding to four different mycobacterial antigens known to confer protection in the HSP65 gene, building a multi-epitope DNA vaccine. After intramuscularly immunization of BALB/c mice, this vaccine generated a specific Th1 response against tuberculosis, with high INF- γ and IL-12 levels, as well as an increase in cytolytic activity due to the known ability of HSP65 to facilitate antigen presentation by MHCI molecules via cross-presentation by dendritic cells.

5. The immune response against mycobacterial infections

5.1. Phagocytes / Antigen presenting cells

Phagocytes and antigen presenting cells (APCs) are responsible for the surveillance of tissues against invading pathogens. These cells are able to recognize PAMPs through specific surface receptors. Pathogens are then internalized, proteolytically processed and their antigens are loaded on MHC molecules and presented to T cells, linking the innate with adaptive immune response. The role of APCs is played by dendritic cells (DCs), macrophages and neutrophils, and in some circumstances also by eosinophils. These cells

are tissue sentinels; they act in association with each other and control and/or potentiate the immune response (Gordon and Taylor 2005). Phagocytes originate from a unique ancestor in the bone marrow. Bone marrow stem cells divide under the effect of cytokines such as IL-1, IL-3 and IL-6, generating a common progenitor of macrophages and granulocytes known as granulocyte-macrophage colony-forming unit (GM-CFU). Another cytokine, the macrophage colony-stimulating factor (M-CSF) induces then the maturation of GM-, which gives rise to monoblasts, pro-monocytes and finally monocytes (Valledor, Borrás et al. 1998).

5.1.1. Neutrophils

Neutrophils are the most abundant leukocytes in the circulation and the first immunological cells to migrate to the infection site thanks to interactions between neutrophil and epithelial cell adhesion molecules. Neutrophil migration is followed by a second wave of cells composed mainly of macrophages. Neutrophils engulf bacteria efficiently and can kill them using antimicrobial machinery consisting of antimicrobial peptides and proteins, proteases, and oxygen and nitrogen reactive species. The short life span of neutrophils somehow limits their role in the control of acute infection. However, their importance is demonstrated by the fact that neutropenia increases substantially the risk of chronic infections. Neutrophils are also able to uptake pathogens in peripheral tissues and transport them to lymphoid tissues, eliciting the activation of T cells through MHC II (Nathan 2006; Appelberg 2007).

After encountering pathogens, neutrophils play a role in initiating adaptive immunity by secreting pro-inflammatory cytokines such as IL-12. Apoptotic neutrophils that had previously ingested and killed bacteria can deliver pathogenic antigens to macrophages and DCs by being engulfed and processed by them, facilitating antigen cross-presentation to T cells. Neutrophil degranulation can enhance the phagocytic ability of macrophages and, moreover, the engulfment of apoptotic neutrophils results in the transfer of antimicrobial

molecules (e.g. antimicrobial peptides) to macrophages, enhancing their killing ability (Silva 2010).

5.1.2. Dendritic cells (DCs)

Human monocytes do not replicate, they leave the bone marrow and enter the circulation. Circulating monocytes are divided in two main populations according to their levels of CD14 and CD16 expression. These surface receptors are involved in the recognition of LPS and of IgG- opsonised pathogens, respectively. CD14^{high}CD16⁻ cells are called classical monocytes since they constitute the major monocyte population in healthy people, whereas CD14⁺CD16⁺ monocytes express higher levels of MHC II and CD32 and are referred to as pro-inflammatory monocytes because they secrete substantial amounts of TNF- α upon TLR agonism. They are believed to be the precursors of DCs because of their ability to migrate across endothelial cell layers, helped by the expression of chemokine receptors and adhesion molecules (Strauss-Ayali, Conrad et al. 2007; Serbina, Jia et al. 2008).

Monocyte subsets can give origin to macrophages or dendritic cells through a differentiation process that is dependent on the milieu. *In vitro* culture of monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) together with IL-4 triggers differentiation into DCs. DCs have a high phagocytic capacity that enable them to acquire, process and present antigens to T and B cells in lymphoid tissues, helped by their high migratory ability. They have a short life-span and are continuously replaced by their blood precursor (Geissmann, Manz et al. 2010). DCs are believed to be the main cell type capable of transporting and presenting antigens to T cells in lymphoid tissues. However, macrophages and neutrophils (Abadie, Badell et al. 2005; Pozzi, Maciaszek et al. 2005) are also able to acquire antigens and migrate to lymphoid tissues. DCs are involved in the cross presentation of foreign antigens, *in vivo*. Cross presentation involves the uptake of exogenous antigens, their association with MHC class I molecules and their

presentation, on the cell surface, to CD8⁺ T cells. The mechanism of cross presentation by DCs, still not completely understood, can generate cytotoxic immunity to viral infections, tumours and in response to DNA vaccination (Heath, Belz et al. 2004; Amigorena and Savina 2010). DCs in peripheral tissues have a high phagocytic capacity, and after being challenged they migrate via lymphatic vessels to the draining lymph node. During their migration they undergo maturation, which involves an enhancement in the expression of MHC II and co-stimulatory molecules that facilitates their interaction with T cells (Hope, Thom et al. 2004). In particular, infection of DCs with mycobacteria elicits initially the secretion of pro-inflammatory cytokines and later the secretion of IL-10, an anti-inflammatory cytokine. IL-10 is a de-activating cytokine that favours intracellular mycobacterial survival and replication, and disease establishment (Beamer, Flaherty et al. 2008). ManLAM present in the mycobacterial cell wall is the main ligand of DC-SIGN, a C-type lectin receptor on the DC surface (Maeda, Nigou et al. 2003), and this seems to be a key DC deactivating event. Geijtenbeek et al. reported that different strains of mycobacterium such as *M. tuberculosis* H37Ra and H37Rv, *M. bovis* BCG, and *M. paratuberculosis*, interact with DC-SIGN via their ManLAM, and that this interaction stimulates the secretion of IL-10 which in turn prevents DC maturation (Geijtenbeek, Van Vliet et al. 2003). IFN- γ activated DCs control *M. tuberculosis* replication but, differently to macrophages, do not kill intra-cellular mycobacteria, therefore these cells can become a reservoir of mycobacteria (Bodnar, Serbina et al. 2001).

5.1.3. Macrophages

Exposure of monocytes to macrophage colony-stimulating factor (M-CSF) induces their differentiation into macrophages, which can then migrate to tissues (Geissmann, Manz et al. 2010). Macrophages participate in the protection against infections, tissue remodelling, coordination of the adaptive immune response and inflammation. They are professional phagocytes that can efficiently internalise particles and antigens, and their endosomal/lysosomal system is able to process from molecules to macromolecular

structures (Harding, Ramachandra et al. 2003). Macrophages show a high degree of heterogeneity, which is reflected in different functions according to the host tissue. Some tissue macrophages such as Langerhans cells in the epidermis, alveolar macrophages in alveoli and microglia cells in the central nervous system can proliferate in situ and maintain the cell population, while in other tissues like bone and liver the replacement of macrophages is from peripheral blood (Gordon and Taylor 2005). Monocyte differentiation is plastic and its fate is under the influence of environmental factors, as reported by Chomarat et al. (Chomarat, Banchereau et al. 2000). These authors observed that differentiation of peripheral blood monocytes into DCs, induced by GM-CSF and IL-4, was reversed by co-incubation with fibroblasts. The monocyte-fibroblast interaction induces the secretion of IL-6 by the fibroblasts, and this cytokine promotes the expression of M-CSF receptor on monocytes. In this way, monocytes can be influenced by the M-CSF they produce themselves. In turn, M-CSF directs monocyte differentiation to macrophages rather than DCs.

Lung alveolar macrophages (AM) constitute the first line of protection against air-borne organisms, including mycobacteria, and play an important role in their elimination (Hope, Thom et al. 2004; Kirby, Raynes et al. 2006; Kirby, Coles et al. 2009). They derive from peripheral blood monocytes that migrate to the lung and differentiate into alveolar macrophages, where they can proliferate sufficiently as to maintain the size of the lung cell population unchanged. Alveolar macrophages express intermediate levels of MHCII and high levels CD11b and CD11c, (MHCII^{int} CD11b^{high} CD11c^{high}) (Strauss-Ayali, Conrad et al. 2007). Unlike their precursor cells, which undergo rapid apoptosis, alveolar macrophages are long-lived and tend to be resistant to apoptotic stimuli. Differentiation into alveolar macrophages results in an increased activity of the pro-survival phosphatidylinositol 3-kinase (PI 3-K)/Akt system and a decrease in the levels of a negative PI 3-K regulator, as well as an increased baseline ROS generation (Flaherty, Monick et al. 2006).

Alveolar macrophages phagocytose apoptotic cells and external pathogens, and have recently been found to transport pathogens to lung draining lymph nodes (Kirby, Coles et al. 2009). They are also involved in tissue remodelling (Smith, Standiford et al. 2007). Alveolar macrophages express high levels of pathogen and opsonization recognition receptors, e.g. CR3, Fc receptors (Reynolds, Atkinson et al. 1975), SR-A (Arredouani, Palecanda et al. 2005), ManR (Zhang, Zhu et al. 2004), and Dectin-1 (Steele, Marrero et al. 2003). In the lung environment, a port of entry for pathogens and particles, the expression of a wide range of receptors by macrophages facilitates an immediate recognition of pathogens that results in a more efficient innate immune response (Smith, Standiford et al. 2007).

Li et al. have compared blood monocytes, AMs and macrophage-CSF-differentiated monocytes (MDMs) from different donors for changes in gene expression (Li, Pritchard et al. 2007). They found that, out of a total of 13,583 probes tested, 161 genes were strongly up-regulated and 210 were strongly down-regulated in AMs compared with MDMs. MHCII(DR α , DQ β 1, DR β 3, DP β 1, DP α 1 and DR β), inhibin bA, the receptor MARCO, the chemokine CCL18, the member of the Ras oncogen family rab4A and a serine proteinase inhibitor can be mentioned among those more expressed in AMs. Among those expressed less in AMs were chitinase 3-like 1, a nuclear factor, GATA-binding protein, pleiotrophin, cyclin D1, cytochrome P450 CYP1B1, the antigen CD36, adenosine monophosphate deaminase and the chemokine CCL3.

5.2. Phagocytosis and the phagosome

Phagocytosis is the first of a sequence of events after pathogen recognition by phagocytic cells. Engulfment involves the isolation of the pathogen in a phagosome, aimed at killing the organism and proteolytically degrade it in order to present antigens at the cell surface and initiate an immunological response. After uptake, the pathogen is confined in a cytoplasmic vesicle called phagosome that immediately starts to acquire new molecules

from the cytoplasm through its fusion with endosomes, in a process called phagosomal maturation. Phagosomes recruit different Rab GTPases according to the maturation stage. In fact, these proteins are involved in the transport control and interaction between membrane-bound cytoplasmic vesicles. Fusion of phagosomes with early endosomes results in the acquisition of early endosomal markers such as Rab5 and early endosome antigen-1 (EEA-1) on the phagosomal surface. In a process termed Rab conversion, Rab5 is exchanged for Rab7, a component of late endosomes. Rab7 recruitment is concomitant with the acquisition of lysosome-associated membrane proteins 1 and 2 (LAMP-1/2), localized in acidic lysosomes (Fig. 2).

The maturation process is accompanied by a gradual acidification of the phagosomal lumen, carried out by recruitment of a vacuolar ATPase proton pump from cytoplasmic vesicles. Concomitantly, activation of the NADPH oxidase superoxide forming system takes place (Rosenberger and Finlay 2003; Blander and Medzhitov 2006; Kinchen and Ravichandran 2008). During the initial phases of a phagosome, its pH is still close to that of the external milieu of the cell and this is favourable to the activity of some bactericidal proteins (Segal, Geisow et al. 1981). A progressive phagosomal acidification is essential for the activity of hydrolases and proteases such as cathepsins, which are delivered to the phagosome at different stages of phagosomal maturation. Garin et al. have isolated latex beads phagosomes and shown that their cathepsins A, D, S, and Z levels change with time (Garin, Diez et al. 2001). The gradual acidification of phagosomes results in a proper processing and presentation of antigens. Lysosomal fusion gives rise to mature phagolysosomes and provides a high degradative capability. In conclusion, processing of the phagosomal cargo ends up in the loading of MHC molecules for antigen presentation and, lastly, to the complete breakdown of the microorganism contained within.

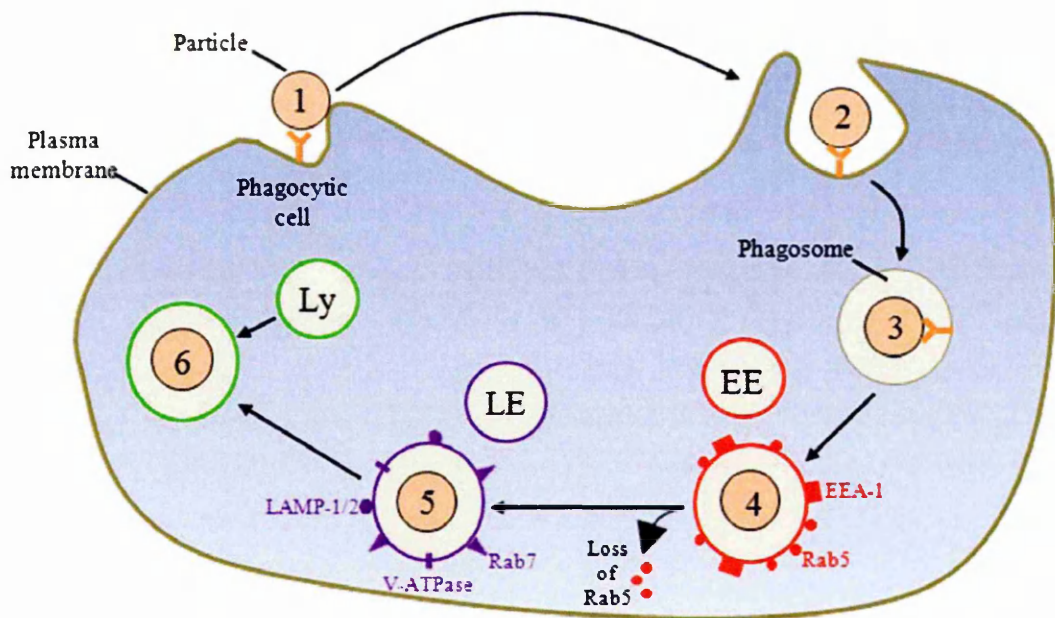


FIGURE 2. Process of phagosome formation and maturation.

The following stages are shown: 1, pathogen recognition by a surface receptor; 2, formation of the phagocytic cup through pseudopode emission; 3, nascent phagosome; 4, early phagosome with its surface markers Rab5 and EEA-1; 5, late phagosome, which after the loss of Rab5 acquires Rab7 and LAMP1 and 2 from late endosomes; 6, phagolysosome, a mature phagosome that fuses with lysosomes, acquiring proteolytic and hydrolytic enzymes. The formation of phagolysosomes is impaired when the pathogen ingested is a virulent mycobacteria.

Reproduced from Steinberg & Grinstein (Steinberg and Grinstein 2008), with modifications.

Rab5 and 7: small GTPase family proteins; V-ATPase: vacuolar-ATPase; EEA-1: early endosomal antigen-1; LAMP1/2: lysosome-associated membrane proteins

EE: Early endosome; LE: Late endosome; Ly: Lysosome.

Proteomic studies of mycobacterial phagosomes have reported that phagosomal maturation is altered by virulent strains of mycobacteria. Maturation is blocked at some stage, preventing the acquisition of late endosomal markers (Rao, Singh et al. 2009). Moreover, these studies open the discussion about ER influence in phagosome formation, based on the finding of several ER proteins in phagosomes. The dynamics of protein expression during phagosomal maturation suggests that the classical model of maturation is over simplistic (Rogers and Foster 2008). Trost et al. have reported the influence of IFN- γ on mouse macrophage activation and phagosomal protein expression, showing the up-regulation of several classical phagosomal markers (Trost, English et al. 2009). They also found that, concomitantly with the effects on phagosomal maturation, IFN- γ delayed the

maturation of early endosomes and could be the reason for the increase in antigen presentation, supported by the presence of MHC molecules in early endosomes.

5.3. Macrophage activation

Circulating monocytes that migrate to tissues and differentiate to macrophages can acquire different activation states after encountering microbial products, cytokines and diverse tissue conditions, which translate into differences in cell phenotypes. The differences concern the expression of surface receptors, antimicrobial activities, antigen presentation and co-stimulation ability (Gordon and Taylor 2005). IFN- γ or LPS induce the so called classical activation of macrophages that results in enhanced microbicidal activity associated with the production of pro-inflammatory cytokines and cell mediated immunity. IL-4 and IL-13, instead, induce an activation associated with tissue repair and humoral immunity, called alternative activation. A third kind of activated macrophages is induced by treating TLR4-primed cells with immune complexes (Gerber and Mosser 2001). These cells become anti-inflammatory and their weak response minimizes tissue damages. They are known as regulatory macrophages.

5.3.1. Classical activation of macrophages

Classically activated macrophages (Ca-M Φ s) are induced by the cytokines IFN- γ and TNF- α . Ca-M Φ s are also named M1 polarized macrophages. These macrophages present enhanced anti-microbial and anti-tumor activities due to their increased capacity to generate reactive oxygen and nitrogen species, and are specifically involved in the protection against intracellular pathogens. Mice deficient in IFN- γ and IL-12 have been reported to be more susceptible to infection by several mycobacterium strains (Jouanguy, Doffinger et al. 1999). They secrete higher amounts of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-12 and IL-23) and chemokines (CCL15, CCL20, CXCL8-11 and CXCL13). The expression of the surface molecules MHCI and II, the co-stimulatory molecules CD80 and CD86 and ICAM-1 (Pestka, Langer et al. 1987; Boehm, Klamp et al.

1997; Zhang, Guo et al. 2003) is up-regulated, which increases the capacity of Ca-MΦs for antigen presentation and migration. Phagocytosis is also enhanced due to the up-regulation of the expression of complement receptor 3 and FcRγI (CD64), which favours the uptake of IgG-opsonised bacteria (Fridman, Gresser et al. 1980; Schroder, Hertzog et al. 2004).

IFN-γ is initially secreted by activated natural killer (NK) cells. That secretion is transient, and IFN-γ generation is later sustained by CD4⁺ Th1 cells, CD8⁺ T cytotoxic lymphocytes, gamma/delta T cells and dendritic cells. IFN-γ has antiviral, immunoregulatory and anti-tumour properties. It up-regulates the transcription of genes involved in pathogen recognition and killing, antigen processing and presentation, apoptosis, trafficking and inhibition of cellular proliferation. It suppresses Th2 cell activity, promotes the adhesion and binding requirements for leukocyte migration, enhances NK cell activity, activates APCs and promotes Th1 differentiation.

Macrophage de-activation occurs due to increased amounts of IL-10 and TGF-β, by a complex process (Schroder, Hertzog et al. 2004; Gordon 2007).

5.3.1.1. IFN-γ signalling pathway

Macrophage activation is induced through a signalling network triggered after binding of IFN-γ to IFN-γR1 on the cell surface (Figure 3). IFN-γR is a transmembrane molecule composed of two IFN-γR1 chains associated with two IFN-γR2 chains (Schroder, Hertzog et al. 2004). Binding of IFN-γ to IFN-γR1 induces conformational changes in the receptor, allowing the interaction of the Janus kinases Jak1 and Jak2 that are already associated with IFN-γR1 and IFN-γR2, respectively. These kinases can then phosphorylate each other, as well as a residue within the cytosolic domain IFN-γR1. This phosphorylated residue works as an anchor for the transcription factor STAT1α, which gets phosphorylated in its C-terminus and forms dimers. These dimers dissociate from the IFN-γR and translocate to the nucleus, where they initiate the transcription of genes through GAS (gamma-activated sequence) promoter elements (Hu, Chen et al. 2007; Gough, Levy et al. 2008), resulting in

the cellular effects of IFN- γ (see sections 5.3 and 5.3.1 above). The importance of this cytokine is illustrated by the failure of IFN- γ -deficient mice infected with *M. tuberculosis* to mount a Th1 response and control the infection (Murray, Young et al. 1998). The IFN- γ signal is physiologically regulated by suppressors of cytokine signalling (SOCS) 1 and 3, which are induced by IFN- γ in a negative feedback, and inhibit the tyrosine kinase activity of Jak1 and 2 (Diamond, Doran et al. 2000). IFN- γ signalling is also affected by mycobacterial infection. *M. avium* is able to down-regulate IFN- γ R through the expression of a transcriptionally inactive STAT1 β , therefore compromising the transcriptional response of macrophages to IFN- γ (Hussain, Zwilling et al. 1999; Alvarez, Zwilling et al. 2003).

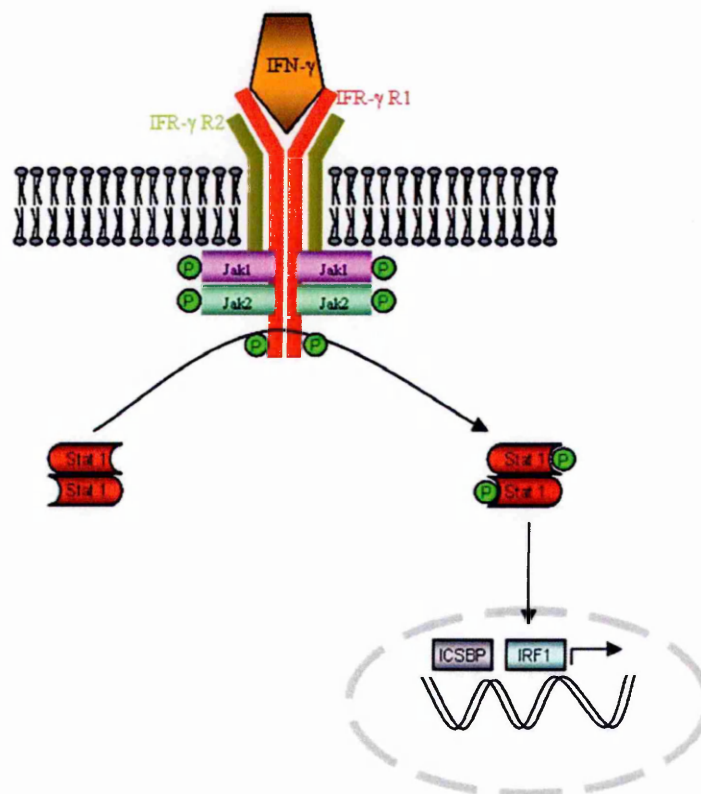


FIGURE 3. IFN- γ signalling pathway. IFN- γ binds to the IFN- γ R1 subunit on the cell surface. The binding induces a change in the morphological conformation of this receptor resulting in inactive Jak2 kinase to be activated by phosphorylation. Jak2 in turn phosphorylates Jak1. Activated Jak1 phosphorylates functionally critical tyrosine residues of each of the IFN- γ R1 chains, creating two adjacent docking sites for Stat1. Stat1 dimers then translocate toward the nucleus where they bind to the IFN- γ -activation promoter site, initiating gene transcription. Negative regulation of gene transcription is done by IRF-2 that antagonizes transcriptional activation of many (IRF-1-inducible) genes by competing for binding sites without promoting gene expression. Extracted from (Takaoka and Yanai 2006), with modifications.

5.3.1.2. Toll-Like Receptors

TNF- α production and secretion by macrophages is elicited after stimulation of TLRs. TLR triggering induces macrophage activation, characterized by the secretion of pro-inflammatory cytokines, enhanced capacity to produce reactive oxygen and nitrogen species and an efficient association with Th1 lymphocyte responses (Yadav and Schorey 2006; Gordon 2007). Apart from TLRs, receptors such as CD14 and dectin-1 can also recognise pathogens and activate macrophages (Gordon 2003).

TLRs are mammalian homologues of the protein coded by the Toll gene of *Drosophila*. They are evolutionary conserved proteins present in vertebrates, as well as in invertebrates. TLR molecular building blocks are represented in bacteria and in plants, and in the latter are known to be required for host defence against infection. The TLRs thus appear to be one of the most ancient, conserved components of the immune system. Nine molecules are operative in humans: TLRs 1-9 (Albiger, Dahlberg et al. 2007; Carpenter and O'Neill 2007). They are transmembrane glycoproteins with an extracellular, leucin-rich domain responsible for PAMP recognition and a cytoplasmic portion homologous to interleukin-1 receptor (IL-1R) (Kaisho and Akira 2002; Jin, Kim et al. 2007). TLR 1, 2, 4, 5, and 6 are expressed on the cell surface and recognise molecules present on the pathogen surface. TLR 3, 7, 8, and 9 are located intracellularly in endosomes and recognize pathogen nucleic acids, for which previous microbe degradation is necessary (McGettrick and O'Neill 2010). Binding of microbial PAMPs to the extracellular domain of TLRs triggers the recruitment of signalling molecules such as the adapter molecule myeloid differentiation primary-response protein (MyD88), with the exception of TLR3 that has TRIF as signalling adaptor; IL-1R-associated kinases (IRAKs); transforming growth factor- β (TGF- β)-activated kinase (TAK1); TAK1-binding proteins (TAB1, TAB2) and tumour necrosis factor-receptor associated factor 6 (TRAF6) (Akira and Takeda 2004) (Fig. 4). The importance of MyD88 in TLR activation is demonstrated by the fact that MyD88^{-/-} mice have a reduced ability to kill *M. avium* (Feng, Scanga et al. 2003). Signalling ends in the

activation of the transcription factor NF- κ B present in the cell cytosol. This involves the phosphorylation of the NF- κ B inhibitor I κ B followed by its ubiquitination and subsequent proteolytic degradation. Free NF- κ B then translocates to the nucleus and promotes the transcription of inflammatory cytokines (see Fig. 4 below). NF- κ B also participates in protecting the host cell against apoptotic stimuli, making possible intracellular mycobacterial growth (Kaisho and Akira 2002; Akira and Takeda 2004; Cristofaro and Opal 2006; Dhiman, Raje et al. 2007).

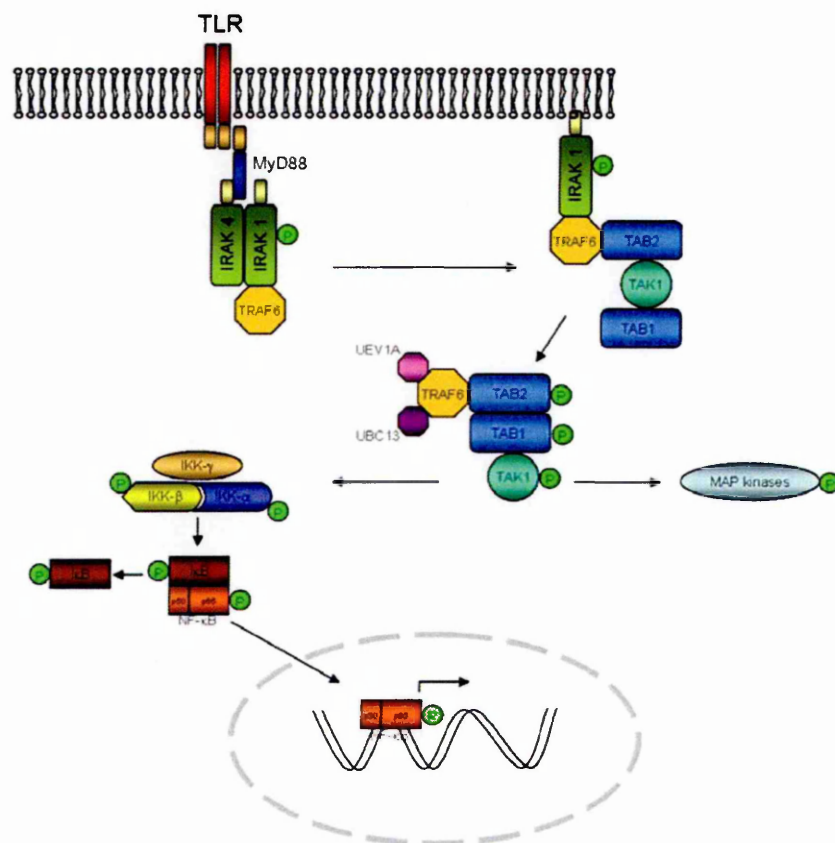


FIGURE 4. TLR2 signalling pathway. TLR2 signalling is triggered by PAMP binding. It starts with the association of MyD88 to the cytoplasmic region of TLR2. MyD88 recruits IRAK4, that associates and phosphorylates IRAK1. Phosphorylated IRAK1 recruits TRAF6 (tumour-necrosis-factor receptor-associated factor 6), and the complex IRAK1-TRAF6 leaves the receptor and associates with TAK1 (transforming-growth factor- β -activated kinase), TAB1 (TAK1-binding protein 1) and TAB2 (TAK1-binding protein 2). This association induces the phosphorylation of TAB2 and TAK1 and the detachment of IRAK1. The complex TRAF6-TAK1-TAB1-TAB2 migrates to the cytosol and associates the two ubiquitin ligases UEV1A and UBC13, which ubiquitinylate TRAF6. In turn, this promotes TAK1 activation. Activated TAK1 phosphorylates MAP kinases and the IKK complex. The IKK complex phosphorylates I κ B, which frees NF- κ B that can then translocate to the nucleus and promote the expression of selected genes, e.g. those of pro-inflammatory cytokines. Extracted from Kumar, H. et al (Kumar, Kawai et al. 2009), with modification.

5.3.2. Alternative activation of macrophages

Alternatively activated macrophages (AaMΦ), also named M2 polarized macrophages or wound-healing macrophages, are involved in the immune response to helminths (Kreider, Anthony et al. 2007) and protozoa (Noel, Raes et al. 2004; Raes, Beschin et al. 2007) and in tissue repair. They are induced by the cytokines IL-4 and IL-13, produced by CD4⁺ Th2 and CD8⁺ T cells, NKT cells, basophils, mast cells and eosinophils in the early stages of tissue damage. AaMΦs show an enhanced expression of SR-A, ManR and MHCII, and secrete more IL-4R, IL-10, IL-1ra, TGF-β and prostaglandin E₂. These secretion products induce a down-regulation of the inflammatory events initiated by Th1 cytokines (Gordon 2003; Ma, Chen et al. 2003; Benoit, Desnues et al. 2008). Puig-Kroger et al have reported that alternative activation increases the expression of DC-SIGN in monocyte-derived macrophages and THP-1 macrophage-like cells, indicating that this receptor is a marker not only of DCs but also of alternative activation (Puig-Kroger, Serrano-Gomez et al. 2004).

AaMΦs express increased arginase-1 but not NOS2 activity. In this way L-arginine is converted to ornithine, a precursor of polyamines and collagen, thus contributing to tissue repair (Gratchev, Guillot et al. 2001; Hesse, Modolell et al. 2001; Gordon 2003). The fact that IL-4 down-regulates nitric oxide generation and up-regulates the expression of transferrin receptor, needed for the iron uptake that is crucial for mycobacterial viability, creates proper conditions for mycobacterial survival (Kahnert, Seiler et al. 2006; Varin and Gordon 2009). In addition, treatment with IL-4 and IL-13 prevents macrophage autophagy induced by IFN-γ, i.e. providing one more element against the control of *M. tuberculosis* infection (Harris, De Haro et al. 2007).

In general terms, intracellular pathogens and allergic diseases result in a shift of immune responses from Th1 to Th2 and in consequence promote AaMΦ phenotypes (Noel, Raes et al. 2004; Raes, Beschin et al. 2007; Fairweather and Cihakova 2009).

5.3.3. Regulatory (Reg) macrophages

Reg macrophages (RegMΦs) are induced by a first priming signal which can be prostaglandins, immune complexes, glucocorticoids, adenine nucleotides, etc, followed by stimulation via TLRs. A change in cytokine secretion pattern, with an overproduction of IL-10 and a shutting off of IL-12 generation, is observed. Therefore, RegMΦs are anti-inflammatory and play an important role in diminishing tissue damage (Mosser and Edwards 2008). Although anti-inflammatory, RegMΦs present functional and biochemical differences compared with AaMΦs. Edwards et al. reported that RegMΦs express low levels of arginase-1 and high levels of NOS2, as well as of MHCII and co-stimulatory molecules (Edwards, Zhang et al. 2006). These data suggest a similarity between RegMΦs and CaMΦs rather than AaMΦs.

5.4. The use of THP-1 cells to study mycobacterium-macrophage interactions

Human macrophage-mycobacteria interactions can be studied using primary cells (blood monocyte-derived macrophages, alveolar macrophages from lung lavages) or *in vitro* differentiated cells. Although the use of primary cells is undoubtedly closer to a physiological situation, they are difficult to obtain in high amounts and suffer from the disadvantage of a considerable variability in cell behaviour between donors.

The THP-1 cell line originates in a monocytic leukemia. These cells become adherent upon treatment with low concentrations of PMA (phorbol 12-myristate 13-acetate) and exhibit many similarities to macrophages (Tsuchiya, Kobayashi et al. 1982).

PMA exerts its effect by activating protein kinase/s that regulate cell proliferation and differentiation (Schwende, Fitzke et al. 1996). Differentiated, macrophage-like cells acquire a macrophage morphology and phagocytic capacity similar to those of monocyte-derived macrophages (Daigneault, Preston et al. 2010).

Kohro et al. reported a number of differences in gene expression between MDMs and PMA-differentiated THP-1 cells, and between non-differentiated and differentiated THP-1

cells. Differentiated THP-1 cells expressed higher levels of CD14, IL-1 β , ManR and scavenger receptor A (SR-A) than undifferentiated ones (Kohro, Tanaka et al. 2004). CD14 expression by PMA-differentiated THP-1 cells was also examined by Daigneault et al, who detected a slight, non-significant increase in the expression of CD14 after differentiation, with these CD14 levels being similar to those of MDMs (Daigneault, Preston et al. 2010).

The expression of some genes was similar in differentiated THP-1 cells and MDMs, e.g. apolipoprotein E, matrix metalloproteinase 9, integrin β 5, CD59, CSF-1, Na, K-ATPase β -1 subunit and transferrin receptor. Some genes involved in innate immunity such as ficolin1, MHC II DR α 1 and interferon γ -inducible protein were expressed by MDMs but not by PMA differentiated THP-1. It is worth noting that Kohro et al. used PMA at a high concentration (200 nM) for differentiation (Kohro, Tanaka et al. 2004).

Differentiated THP-1 cells and MDMs have been found to bind to a similar extent opsonised and non-opsonised *M. tuberculosis*, a reflection of the fact that they express equivalent amounts of *M. tuberculosis* binding receptors, but they differ in the expression of glucan receptor, responsible for yeast recognition (Stokes and Doxsee 1999). These authors also observed that both cells allowed the intracellular replication of *M. tuberculosis*. Additionally, Isoniazid was active against intra-cellular mycobacteria in both cells, indicating that both constitute viable models for intra-cellular activity studies (Stokes and Doxsee 1999).

Transcriptional studies showed that THP-1 macrophage-like cells and AMs regulated different apoptotic genes in response to *Bacillus anthracis* spore challenge. Nevertheless, both cells were able to mount a pro-inflammatory response and secrete high amounts of TNF- α (Bradburne, Chung et al. 2008; Dozmorov, Wu et al. 2009). Moreover, THP-1 macrophages were found to resist entering apoptosis after infection by virulent *M. tuberculosis* and *M. bovis* strains, like alveolar macrophages (Riendeau and Kornfeld

2003). Dhiman et al. showed later that the resistance of THP-1 macrophages to apoptosis is mediated by a long term NF- κ B activation (Dhiman, Raje et al. 2007).

5.5. Killing mechanisms of macrophages

After being recognised and internalised by macrophages, invading pathogens are confined inside phagosomes formed by a plasma membrane invagination, with which cytoplasmic endosomes and lysosomes gradually fuse. Microbial killing is induced by lysosomal antimicrobial proteins (Martin, Ganz et al. 1995; Houghton, Hartzell et al. 2009) and by reactive oxygen species, nitric oxide and derived molecules (Miller and Britigan 1997; Storz and Imlay 1999). Oxygen and nitrogen metabolites are generated by enzymes or protein complexes present in the plasma membrane of phagocytes and also in the plasma membrane-derived phagosomal membranes. These enzymes are barely active in resting cells (basal activity) and are activated during the process of pathogen recognition and uptake. Activation is favoured by inflammatory cytokines such as IFN- γ and TNF- α .

Oxygen metabolites (reactive oxygen species, ROS) are generated by oxygen reduction, catalysed by the NADPH oxidase complex, and nitric oxide is formed from arginine through the activity of nitric oxide synthase (NOS2) (Babior 2000) (see reactions in Figs. 5 and 7, sections 5.5.1.1 and 5.5.2). Since the active molecules generated can be damaging to the phagocyte itself and their surroundings, their levels are regulated by scavenging enzymes: superoxide dismutases (SOD), hydrogen peroxide-degrading catalases and arginine-degrading arginases. Oxygen and nitrogen metabolites exert their microbicidal action by reacting with key bacterial molecules. They oxidize membranes and enzymes, induce DNA damage and mutagenesis, and produce inhibition of membrane transport processes (Miller and Britigan 1997; Imlay 2003).

5.5.1. Superoxide/ROS formation and degradation

5.5.1.1. NADPH oxidase

This enzyme complex is in a dormant stage in resting professional phagocytes and after becoming active catalyses the synthesis of superoxide (O_2^-) from O_2 and NADPH (Babior 2000), which leads to the generation of ROS (Fig. 5). The NADPH oxidase is composed of different proteins distributed between the cell membrane and cytosol. Ligation of receptors on the phagocyte or phagosome membrane triggers the assembly of the NADPH oxidase components at or close to the receptors site and oxygen reduction is switched on. Superoxide anion, a long-lived oxygen radical, begins to be generated on the external side of the cellular membrane and/or on the luminal side of the phagosomal membrane, i.e. where the engulfed pathogen is present. The membrane components of the NADPH oxidase are gp91^{phox} and p22^{phox}, closely interacting with each other. Gp91^{phox} is the catalytic protein in charge of the electron transfer from NADPH toward O_2 . The cytosolic components of the NADPH oxidase are p47^{phox}, p67^{phox} and p40^{phox}, which associate with the small G protein Rac1 (Babior 2004). As a consequence of cell activation (receptor ligation), the cytosolic NADPH components translocate toward the membrane and assemble with the membrane-bound components to form the active oxidase complex. Translocation is driven by p47^{phox}, which becomes strongly phosphorylated on serine residues by the action of different protein kinases, after cell activation. This induces conformational and affinity changes that result in the translocation of the complex and the activation of its catalytic activity (Figure 5) (El-Benna, Dang et al. 2008; El-Benna, Dang et al. 2009).

The relevance of oxygen metabolites in the protection against pathogens is illustrated by the situation in patients with chronic granulomatous disease. These subjects fail to activate the NADPH oxidase for different reasons according to the disease variant and, as a consequence, do not produce ROS. The result is a strong impairment in the clearance of

many bacterial and fungal pathogens which often ends in premature death (Segal, Romani et al. 2009).

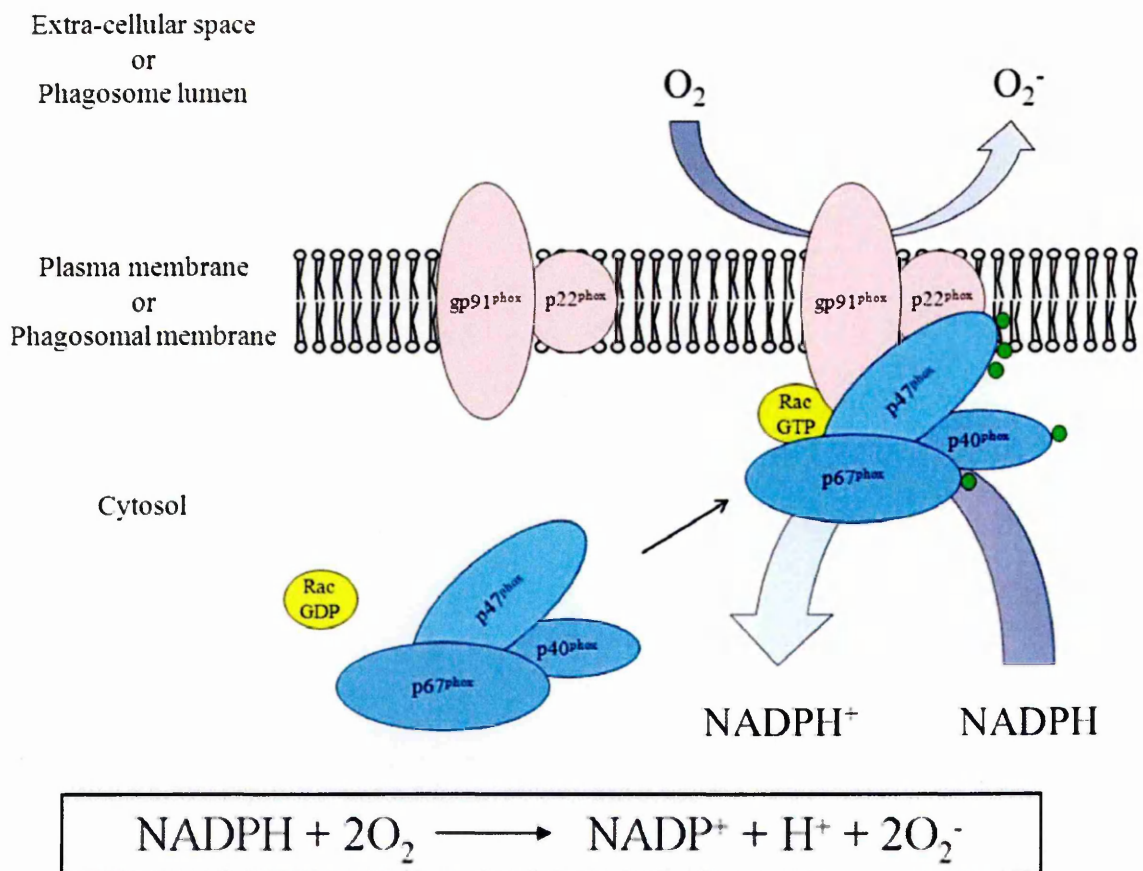


FIGURE 5. NADPH oxidase complex assembly and superoxide generation. In resting cells, the components of the NADPH oxidase are distributed between the cytosol (p67^{phox}, p47^{phox}, p40^{phox}, Rac) and the membrane (gp91^{phox} and p22^{phox}). After receptor ligation, the cytosolic components are phosphorylated and translocate to the membranes where they associate with gp91^{phox} and p22^{phox}, constituting the active NADPH oxidase. The activated NADPH oxidase uses cytosolic NADPH to reduce oxygen to superoxide anion, either on the external side of the plasma membrane or within the phagosome, according to the site of receptor ligation. Extracted from El-Benna, J. et al (El-Benna, Dang et al. 2008), with modifications.

5.5.1.2. Superoxide dismutases

In order to control the negative effects of an excessive production of ROS, eukaryotic cells scavenge the O₂⁻ generated by the NADPH oxidase by means of superoxide dismutases (SODs) that catalyse the conversion of O₂⁻ to H₂O₂ and O₂ (see Fig. 6 below).

Dismutation of O_2^- can take place spontaneously but slowly, and SODs accelerate the reaction by 10^4 -fold (Forman and Torres 2002). Two dismutases have been identified, the cytosolic, constitutive CuZnSOD and the mitochondrial, inducible MnSOD (Babior 2000). The H_2O_2 generated is bactericidal only at very high concentrations, and is quite toxic to cells. Catalase converts H_2O_2 to H_2O and O_2 (Fig. 6), completing the cellular protection system (Forman and Torres 2002).

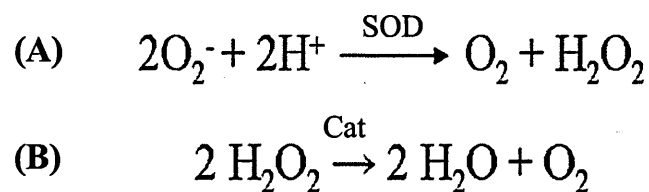


FIGURE 6. Superoxide dismutase and peroxidase reactions (protection against oxidative stress).

A. Superoxide dismutases catalyse the dismutation of superoxide anion in hydrogen peroxide and oxygen.

B. Catalase converts hydrogen peroxide in water and oxygen.

SOD : Superoxide dismutase; Cat : catalase.

5.5.2. Nitric oxide generation

The generation of nitric oxide (NO) is catalysed by NOS2 or iNOS, a soluble, inducible enzyme that converts L-arginine in L-citrulline and NO (see Fig. 7 below). The production of NO by macrophages is regulated by cytokines. Pro-inflammatory cytokines (IFN- γ , IL-1 β and TNF- α) up-regulate the expression of NOS2, increasing NO synthesis. The potent anti-inflammatory cytokines IL-10 and TGF- β inhibit NO generation, favouring the survival of intracellular pathogens. The expression of NOS2 is down-regulated when macrophages are alternatively activated by IL-4 and IL-13.

NOS2 activity is antagonized by arginase-1, which catalyses the hydrolysis of L-arginine into urea and ornithine (Fig. 7). The activity of both arginase-1 and NOS2 is dependent on L-arginine availability (Wanaseen and Soong 2008; Pesce, Ramalingam et al. 2009), but the interplay between NOS2 and arginase-1 is not restricted to a competition for the same substrate. Intermediates of NO synthesis like N^G -Hydroxy-L-arginine (NOHA)

are able to inhibit arginase-1 activity while, on the other hand, arginase-1 can inhibit the expression of NOS2 at translational levels (Wu and Morris 1998).

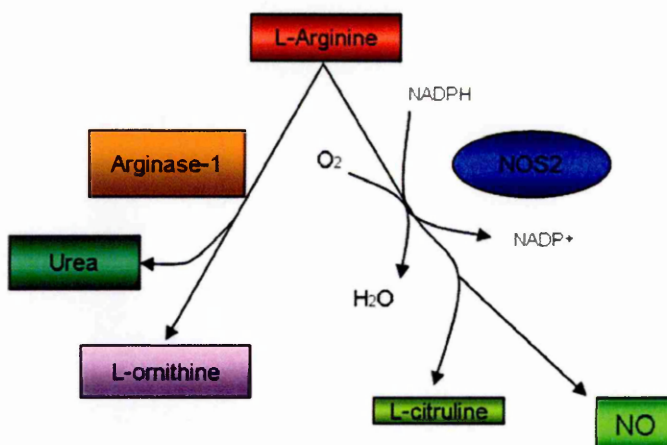


FIGURE 7. L-arginine metabolism. L-arginine is the substrate for two different enzymatic pathways. Classical activation of macrophages by IFN- γ and TNF- α induces the expression of nitric oxide synthase 2 (NOS2) that uses L-arginine to generate nitric oxide (NO) and promote bacterial killing. Instead alternative activation of macrophages by IL-4 and IL-13, and the anti-inflammatory cytokine IL-10, induces the expression of arginase-1 that competes with NOS2 for L-arginine, inducing the generation of L-ornithine and collagen formation for tissue repair. Adapted from Gordon, S. (Gordon 2003).

NO is bactericidal at high concentrations. The effects of NO at low concentrations are more likely to be mediated by peroxynitrite (ONOO⁻), a reactive molecule generated by reaction of NO with O₂⁻ (Figure 8). ONOO⁻ is a strong oxidant, its target molecules being deoxyribose, lipids and proteins (Bartosz 1996).

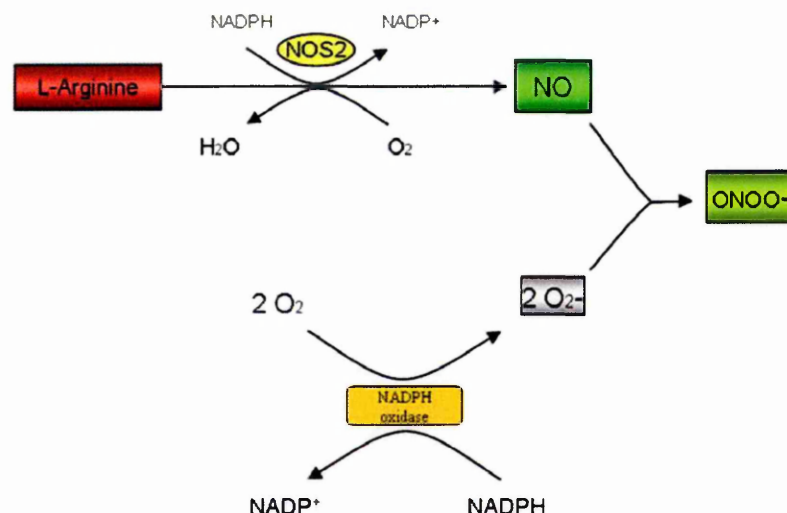


FIGURE 8. Peroxynitrite anion generation. Superoxide and nitric oxide are both generated in the phagosome lumen, thus having the opportunity of reacting to generate an active product, peroxynitrite (OONO₂⁻). Reproduced from Fang, F.C. (Fang 2004).

6. Binding receptors involved in macrophage recruitment, signalling and internalisation.

An effective immune response depends on the fast recognition and elimination of pathogens. The presence on the cell surface of more or less specific receptors that recognise different PAMPs enables phagocytes to detect and deal with a wide range of different pathogens. Mycobacteria express PAMPs that can be recognised simultaneously by macrophages, involving opsonization or not. It is therefore difficult to establish which receptor/s play more important a role. In fact, an efficient immune response may involve the engagement of more than a single receptor. For instance, trehalose-6,6'-dimycolate (*M. tuberculosis* cord factor) recognition and triggering of cell signalling is mediated by MARCO, TLR2 and CD14 (Bowdish, Sakamoto et al. 2009). Some ligand molecules can be recognised by more than a receptor on the phagocyte surface. In addition, the expression of receptors changes according to the activation state of macrophages, i.e. the milieu and tissue where they are. There is also the phenomenon of receptor cross-regulation, commented in section 6.5 below regarding FcγRs and CRs. In any case, the route of entry of a microorganism, in combination with the regulatory factors present in the external milieu, will determine what signal transduction mechanism/s is/are activated and what type

of host cell response will take place, with direct implications for bacterial survival (Ernst 1998).

6.1. Complement Receptor 3

The complement receptor 3 (CR3, $\alpha_M\beta_2$, CD11b/CD18 or Mac-1) is a heterodimeric surface receptor that together with lymphocyte function-associated antigen-1 (LFA-1) ($\alpha_L\beta_2$ or CD11a/CD18), CR4 ($\alpha_X\beta_2$ or CD11c/CD18) and $\alpha_D\beta_2$ is part of the integrin superfamily, with a common β_2 subunit. Integrins are transmembrane proteins involved in cell-cell adhesion, phagocytosis, chemotaxis and cell activation (Hogg and Leitinger 2001; Velasco-Velazquez, Barrera et al. 2003). Activation by inflammatory mediators increases not only the surface expression of CR3 but also its adherence capacity, through the induction of conformational changes (Jones, Knaus et al. 1998).

CR3 is a promiscuous receptor that can recognize a wide variety of molecules, therefore it is important for the internalisation and elimination of several pathogens (Agramonte-Hevia, Gonzalez-Arenas et al. 2002; Mobberley-Schuman and Weiss 2005). Mycobacteria bind to CR3 after iC3b coating (opsonisation) or via the recognition of polysaccharides on its surface by the CR3 lectin domain (non-opsonic phagocytosis) (Mueller-Ortiz, Wanger et al. 2001). The importance of CR3 regarding the binding and internalization of mycobacteria was demonstrated by Stokes et al., who observed a significant reduction in *M. tuberculosis* binding after specific blocking of CR3 receptor with an antibody (Stokes and Doxsee 1999). However, CD18-deficient mice were found to be able to phagocytose *M. avium*, suggesting that this pathogen can be internalized by receptors other than CR3 (Bermudez, Goodman et al. 1999).

Phagocytosis through CR3 is initiated after pathogen binding, which induces the phosphorylation of its β -chain. This leads to the association of CR3 intracellular domains with the cytoskeleton, resulting in phagocytic cup formation. Gaining entry through CRs is important for the fate of intracellular pathogens since it does not induce a respiratory burst and results in a decreased synthesis of TNF- α and IL-12 by macrophages (Ehlers 2000;

Irani and Maslow 2005), therefore being favourable to mycobacterial proliferation (Wright and Silverstein 1983).

6.2. Mannose Receptor

Mannose receptor (ManR) is a transmembrane protein belonging to the C-type lectin family that binds to mannose and fucose through its lectin-like carbohydrate recognition domain (Stahl and Ezekowitz 1998). ManR interacts with mannose-capped lipoarabinomannan (ManLAM) molecules of the mycobacterial cell wall, and this is a favourable route of entry for mycobacteria since a respiratory burst is not induced (Kang and Schlesinger 1998; Astarie-Dequeker, N'Diaye et al. 1999; Torrelles, Azad et al. 2006). Moreover, the ManR-dependent internalisation of heat-killed *S. aureus* or of beads coated with *M. avium* cell wall glycopeptidolipids containing mannose delays phagosome maturation (Shimada, Takimoto et al. 2006; Sweet, Singh et al. 2010). The importance of ManR was established by inhibition assays using mAb against ManR, which reduced the binding of virulent *M. tuberculosis* strains to monocyte derived macrophages by approximately 50% (Schlesinger 1993).

6.3. CD14

CD14 is a glycoprotein anchored to the plasma membrane of mononuclear phagocytes. It is a receptor for lipopolysaccharide (LPS) from Gram-negative bacteria and lipoarabinomannan and phosphatidylinositol-anchored lipoglycan on mycobacteria cell walls (Wright 1995). CD14, associated to TLR4 and to a non-membrane-bound myeloid differentiation protein 2 (MD-2), forms a surface complex that recognises LPSs captured by soluble LPS binding protein (LBP). In this complex, the recognition of LPS is done by CD14, whereas TLR4 activates signal transduction, which in turn elicits the activation of NF- κ B followed by the secretion of pro-inflammatory cytokines such as TNF- α and IL-1 β (Chow, Young et al. 1999; Takeuchi, Hoshino et al. 1999; Thorgersen, Pharo et al. 2009). CD14 also plays a role in the uptake of non-opsonized mycobacteria by human THP-1

macrophage-like cells and by microglia, which is a LBP-dependent event (Peterson, Gekker et al. 1995; Schiff, Kline et al. 1997). Although CD14 alone can induce mycobacterial uptake, optimal internalisation is achieved when CD14, in combination with TLR2, triggers signalling leading to a more active CR3 (Sendide, Reiner et al. 2005).

6.4. Scavenger receptors

Scavenger receptors (SRs) are a group of transmembrane glycoproteins that bind and uptake chemically modified low density lipoproteins (LDL), apoptotic cells, and pathogens. These receptors have been divided in different classes according to their structure and ligand affinities (Gough and Gordon 2000; Palecanda and Kobzik 2001), with the molecules in the SR-A group being those with binding affinity for pattern recognition molecules (Gordon 2002). The SR-A family is composed by SR-AI, SR-AII and MARCO (Macrophage Receptor with Collagenous structure). The first two are expressed by the same gene, with the differences being due to mRNA splicing, while MARCO is expressed by a different gene. These SRs recognize trehalose dimycolate or Cord Factor (CF) from *Mycobacterium tuberculosis*, lipoteichoic acid (LTA) from Gram-positive and LPS from Gram-negative bacteria as their PAMPs, in an opsonin-independent way (Haworth, Platt et al. 1997; Kraal, van der Laan et al. 2000). While the binding of LPS to CD14 is a mediator of macrophage activation and elicits the secretion of pro-inflammatory cytokines (TNF- α , IL-6, IL-12 and IL-1 β), its binding to SR-A does not result in TNF- α or IL-12 secretion. This suggests that SR-A may be playing a protective role regarding excessive responses, by removing any surplus of circulating LPS (Haworth, Platt et al. 1997; Jozefowski, Arredouani et al. 2005; Ozeki, Tsutsui et al. 2006). Instead, the synergic combination of MARCO, TLR2 and CD14 does result in macrophage cytokine response to trehalose-dimycolate (Bowdish, Sakamoto et al. 2009).

6.5. Fcγ receptors (FcγRs)

FcγRs are a group of transmembrane proteins that bind the Fc domain of IgG. Three classes of receptors have been identified, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), encoded by different genes. These receptors are involved in phagocytosis, endocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) and antigen presentation. FcγRI (CD64) is expressed in monocytes and macrophages, binds the Fc portion of monomeric IgG with high affinity and its expression is up-regulated by pro-inflammatory cytokines such as IFN-γ and IL-12. FcγRII (CD32) and FcγRIII (CD16) are widely expressed by innate immune cells and recognize only multimeric IgG since they have low affinity for IgG (Raghavan and Bjorkman 1996; Dijstelbloem, van de Winkel et al. 2001; Gerber and Mosser 2001; Davis, Olsen et al. 2006). FcγRs promote the endocytosis of small antigen-IgG complexes, enhancing in this way antigen processing and presentation by MHC II molecules (Raghavan and Bjorkman 1996; Ravetch and Bolland 2001; Ortiz-Stern and Rosales 2003). FcγR structure comprises an extracellular domain that recognises the Fc domain of IgGs, a transmembrane domain and a cytosolic tail that is crucial for signalling. The latter is based on tyrosine phosphorylations, in the case of FcγRII variant A, or on interactions with small transmembrane proteins that contain phosphorylation sites. FcγRII variant B works as an inhibitory receptor through tyrosine-containing inhibitory motifs recruiting phosphatases that down-modulate signals by other FcγRs (Gerber and Mosser 2001; Park 2003). Phagocytosis elicited by FcγRs is characterized by the formation of a phagocytic cup after activation of Src family kinases and casein kinase II, which are important for actin polymerization (May and Machesky 2001). FcγRs are also able to interact with the β2-integrin subunit of complement receptors and modulate their activity (Ortiz-Stern and Rosales 2003).

7. Antigen presentation

The innate immune response is extended by secretion of pro-inflammatory cytokines and by presentation of antigens to T cells, which initiates the adaptive immune response. This relies on signals induced by the major histocompatibility complex (MHC) and co-stimulatory molecules, in the immunological synapse.

Naïve T cell activation requires two distinct signals in the immunological synapse, the main one being antigen recognition by interaction of the TCR complex (T cell receptor plus its co-receptor CD4⁺ or CD8⁺) with MHCI or II molecules. This interaction is critical to maintain the specificity of the immune response. The second signal is associated with co-stimulation, it is antigen-independent and is required for sustained T cell proliferation. The absence of co-stimulation leads to a state of T cell unresponsiveness (Bromley, Burack et al. 2001). Co-stimulation involves the binding of molecules of the B7 family (CD80 and CD86) and CD83, expressed by APCs, to CD28 on the T cell surface. This interaction is crucial for T cell differentiation in either CD4⁺ or CD8⁺. Differentiation is followed by expansion and secretion of cytokines such IL-1, IL-2, IL-4, IL-5, IFN- γ , TNF- α . At a later activation stage, counter-receptor cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152) is recruited to the immunological synapse. CTLA-4 shares the same ligands with CD28, but it antagonises CD28 by eliciting an inhibitory signal that works as a negative feedback in the regulation the T cell activation levels (Lenschow, Walunas et al. 1996; Greenwald, Latchman et al. 2002). Other receptors have also been described as co-stimulatory, e.g. ICAM-1 (Zuckerman, Pullen et al. 1998). ICAM-1 is important for the stabilization of the immunological synapse by binding LFA-1 on the T cell surface (Boisvert, Edmondson et al. 2004).

The cellular junction of the synapse is initially composed of LFA-1 on the T cell surface and ICAM-1 on the APC surface, which provides a base for the subsequent interaction between TCR on the T cell and MHC-antigen complexes on the APC surface. The complex TCR-MHC-antigen moves to the central part of the synapse through an actin-

dependent mechanism, constituting the so called central supramolecular activation cluster (SMAC). This central SMAC is surrounded by a peripheral SMAC composed of LFA-1–ICAM-1 complexes that confers stability to the synapse (Grakoui, Bromley et al. 1999). The additional interaction of co-stimulatory molecules located on both sides, i.e. CD80, CD86 and CD83 on T cells and CD28 on APCs, provides signals for actin rearrangement, immunological synapse maturation, and T cell activation (Samstag, Eibert et al. 2003; Wabnitz, Kocher et al. 2007).

7.1. Molecules responsible for the immunological synapse on the APC

7.1.1. Major Histocompatibility class I molecules (MHCI)

MHCI molecules are expressed by different cell types. They are responsible for the priming of naïve CD8⁺ T cells, initiating the cytotoxic immune response and the immune response against viral infections and tumors (Raghavan, Del Cid et al. 2008).

The MHCI heavy chain is a transmembrane glycoprotein that associates to the chaperone calnexin on the ER membrane and undergoes folding and disulfide bond-related conformational changes. The re-arranged heavy chain, together with β_2 microglobulin (β_2m), is then incorporated into the peptide-loading complex involving TAP (transporter associated with antigen processing, composed of 2 subunits), tapasin, calreticulin, and the thiol oxidoreductase ERp57. Peptides generated in the cytosol are transported to the ER by TAPs and bind to the heavy chain of MHCI, i.e. they are loaded on the MHCI- β_2m complex. The MHCI-peptide complex then leaves the ER and reaches the plasma membrane through Golgi apparatus, where it can be recognized by CD8⁺ T cells (Cresswell, Ackerman et al. 2005).

The presentation of foreign antigens is generally accepted to take place via MHCII molecules (section 7.1.2). However, APCs, especially dendritic cells, can acquire foreign antigens by phagocytosis or receptor-mediated internalization and present them associated

with MHCI in a process called cross-presentation. Cross-presentation involves the transfer of internalized proteins from the phagosome to the cytosol, followed by proteolysis and loading on MHCI in the ER (Shen and Rock 2006).

Mycobacteria replicate within host cell phagosomes and prevent their maturation into phagolysosomes (Flynn and Chan 2001). Virulent strains of mycobacteria have been reported to be able to translocate to the cytosol contemporarily with the beginning of intra-phagosomal replication (van der Wel, Hava et al. 2007). Once in the cytosol, these bacteria can release some of their proteins, which are then proteolytically degraded. The corresponding peptides are transported to the ER by TAPs and loaded on MHCI molecules to be presented. In this way, mycobacteria can elicit cross-priming of CD8⁺ T cells (Weerdenburg, Peters et al. 2010).

7.1.2. Major Histocompatibility class II molecules (MHCII)

MHCII molecules are receptors expressed constitutively by antigen presenting cells (APCs), while MHCI is more widely expressed. They are responsible for the antigen-specific activation of CD4⁺ T cells that recognise MHC-antigen complexes through their T-cell receptor (TCR). MHC II expression is modulated by IFN- γ , and this modulation can be modified by several other stimuli such as IL-4, TNF- α , TGF- β , IFN- α and β . Cell-cell interactions can also regulate MHCII expression, as well as infection with *M. tuberculosis*, which is inhibitory (Noss, Pai et al. 2001).

MHCII is a group of heterodimeric glycoproteins consisting of two non-covalently-linked subunits, α (33-35 kDa) and β (25-30 kDa). The $\alpha\beta$ dimer is assembled in the ER to include a third molecule, an invariant chain called Ii. After proteolysis of Ii in the $\alpha\beta$ -Ii complex, the $\alpha\beta$ dimer becomes associated with the CLIP peptide. This association protects the complex until it reaches the endosome or lysosome, where CLIP is exchanged for antigenic peptides. Finally, the complex MHC-antigen is transported to the cell membrane, where it can be recognised by the TCR of CD4⁺ cells (Cresswell 1994; Mach,

Steimle et al. 1996; Pieters 1997; Villadangos 2001; Torres, Ramachandra et al. 2006; Rocha-Ramirez, Estrada-Garcia et al. 2008).

7.1.3. Co-stimulatory molecules (B-7 family, CD83, ICAM-1)

These molecules are receptors responsible for the second signal that promotes T cell activation together with that induced by MHCI and II. They are critical for T cell activation, and their absence results in anergy of these cells. The interactions between APC and T cells are antigen independent and involve the co-stimulatory molecules B7.1 (CD80), B7.2 (CD86) and CD83 as well as adhesion molecules like ICAM-1 and CD58 on the APC surface, and their corresponding ligands CD28, CTLA-4, LFA-1 (CD11a/CD18) and CD2 on the T cell surface counterpart (Mohagheghpour, Gammon et al. 1997; Rajavelu and Das 2008).

7.1.3.1. B7 family

The B7 family is composed of B7.1 (CD80) and B7.2 (CD86). CD86 is constitutively expressed and is up-regulated immediately after MHC binding to TCR, while CD80 is weakly expressed in resting cells and its expression increases gradually with T cell activation. This reflects the different roles played by these receptors in the immune response: CD86 is crucial to initiate it, while CD80 is important for sustaining T cell activation (Vincenti 2008). CD80 and CD86 bind to the same ligand on T CD4⁺ cells. Initially they interact with the constitutively expressed CD28, leading to the activation of the T lymphocytes. Successively, CD28 levels are down-regulated and those of its homologous CTLA4 increase. CTLA4 has a high affinity for CD80 and CD86 but, differently to CD28, dampens the activation of T cells, keeping a balance in the immune response (McAdam, Schweitzer et al. 1998). Studies using blocking mAb against CD80 and CD86, in mice, have demonstrated the importance of these receptors for CD4⁺ T cell expansion and activation (Lang, Nguyen et al. 2002). The expression of CD80 and CD86 is

negatively affected by *M. tuberculosis* infection, which could compromise T cell activation (Kan-Sutton, Jagannath et al. 2009).

7.1.3.2. CD83

CD83 is directly involved in the modulation of antigen presentation, with its expression being correlated with CD86 and MHCII. During antigen presentation and immunological synapse formation, CD83 binds to its ligand on the T cell surface. Studies using CD83-deficient mice concluded that CD83 is critical for the generation CD4⁺ T cells (Fujimoto, Tu et al. 2002; Garcia-Martinez, Appleby et al. 2004). CD83 expression is well established in dendritic cells. It is also present in intracellular compartments of monocytes and macrophages, and translocates to the cell membrane upon activation by LPS (Cao, Lee et al. 2005; Klein, Koch et al. 2005; Breloer and Fleischer 2008).

7.1.3.3. ICAM-1 (CD54)

Intercellular Adhesion Molecule-1 (ICAM-1) is a transmembrane glycoprotein and a member of the immunoglobulin family with five Ig-like domains. It is expressed constitutively at low levels in leukocytes, and is up-regulated by pro-inflammatory cytokines such as IL-1 β , TNF- α and IFN- γ or by lipopolysaccharide (LPS). ICAM-1 mediates cell-cell and cell-matrix interactions. CR3 (CD11b/CD18) is recognized by the third Ig-like domain of ICAM-1, and LFA-1 (CD11a/CD18) by the first Ig-like domain. These interactions play an important role in leukocyte migration and in the activation of T cells, where it acts as a co-stimulatory molecule (Miller, Knorr et al. 1995; Fisher, Lu et al. 1997; Roebuck and Finnegan 1999; Hubbard and Rothlein 2000).

Infection with *M. avium* down-regulates ICAM-1 expression in macrophages (van de Stolpe and van der Saag 1996; Mohagheghpour, Gammon et al. 1997; Thornton and McDaniel 2005). This could affect antigen presentation and the initiation of the adaptive immune response.

8. Cytokine secretion

Cytokines are fine regulators of macrophage activation. They control macrophage responses in order to achieve a satisfactory clearance of pathogens and, on the other hand, to avoid excessive inflammation (Hu, Chakravarty et al. 2008).

After being challenged by pathogen molecules through TLRs, innate immunity cells (neutrophils, NK cells and macrophages) become activated and inflammation is promoted (Danelishvilli and Bermudez 2003). This active phase is characterized by the secretion of type 1 cytokines such as IL-1 β , TNF- α and IL-12 (Wang, Wakeham et al. 1999).

IL-12 plays an important role in the protection against intracellular pathogens by promoting and maintaining a Th1 response. IL-12 is able to induce the production of IFN- γ by T and NK cells. Both TNF- α and IFN- γ will activate macrophages and increase their bactericidal activity (Brombacher, Kastelein et al. 2003; Trinchieri 2003; Langrish, McKenzie et al. 2004). Studies with mice deficient in the p40 subunit of IL-12 (IL-12 p40^{-/-}) have confirmed that this cytokine is critical for the control of mycobacterial infection through the induction of IFN- γ secretion (Cooper, Magram et al. 1997; Cooper, Kipnis et al. 2002).

IL-1 β is produced as an inactive pro-form after TLR stimulation of macrophages, and becomes active after a proteolytic cleavage involving the inflammasome (Martinon, Burns et al. 2002). *M. tuberculosis* infection has been reported to prevent inflammasome activation and therefore IL-1 β activation could be compromised in infected macrophages (Master, Rampini et al. 2008). The cytoplasmic domain of the IL-1 β receptor, IL-1R, is homologous to that of TLRs, and provides the link to a signal transduction leading to the activation of NF- κ B. This results in the secretion of pro-inflammatory cytokines, i.e. in macrophage auto-activation (Kontny, Ziolkowska et al. 1999; Sutterwala, Ogura et al. 2007; O'Neill 2008; Netea, Nold-Petry et al. 2009).

TNF- α is an important macrophage autocrine activating cytokine. It plays a central role in controlling infection by inducing apoptosis of infected cells and promoting the

generation of oxygen radicals and nitric oxide. Contemporarily, and in order to protect the cells against the deleterious effects of oxygen metabolites, TNF- α increases the expression of mitochondrial Mn-superoxide dismutase (MnSOD) without affecting that of cytosolic CuZnSOD (Wong 1995). In mycobacterial diseases, TNF- α contributes to granuloma formation and therefore to the control of infection (Kwon 1997; Okamoto, Fujita et al. 2006). Its role in tuberculosis control is illustrated by the effects of TNF- α blocking therapies employed in the treatment of Crohn's disease and rheumatoid arthritis. In fact, these therapies have been found associated with an increase in pulmonary tuberculosis (Keane, Gershon et al. 2001; Mayordomo, Marengo et al. 2002).

The macrophage-activating effects of the IFN- γ secreted by T and NK cells are discussed in section 5.3.1 (Classical activation of macrophages).

In order to balance the immune response and avoid tissue damages, type 2 cytokines (IL-4, IL-6, IL-10, TGF- β) are secreted, that counteract the pro-inflammatory activities elicited by infection (Opal and DePalo 2000).

IL-6 is a pleiotropic cytokine expressed during the acute-phase of infection, showing anti-inflammatory effects (Opal and DePalo 2000). It down-regulates the expression of the pro-inflammatory cytokines IL-1 β and IFN- γ and decreases the expression of TNF- α receptors, therefore decreasing the cellular response to TNF- α . It stimulates the secretion of anti-inflammatory molecules such as IL-1R antagonist (IL-1Ra) and soluble TNF- α R, while it does not affect the secretion of IL-10 and TGF- β . In addition, IL-6 also impairs antigen presentation by infected macrophages, which limits T cell activation (Xing, Gauldie et al. 1998; Danelishvili and Bermudez 2003).

IL-10 is an important anti-inflammatory cytokine that tunes the immune response, limiting its duration and intensity, hence diminishing tissue damage (Herrero, Hu et al. 2003). It is produced by Th1 and Th2 cells, B cells, monocytes, macrophages and dendritic cells, after stimulation. TLR2/4 agonism is a potent inducer of IL-10 synthesis. IL-10 inhibits antigen presentation by APCs through the down-regulation of the expression of

MHCII molecules as well as the co-stimulatory receptors ICAM-1, CD80 and CD86. It decreases the secretion of IFN- γ by Th1 cells, and arrests the secretion of the pro-inflammatory cytokines IL-1, IL-6, IL-12 and TNF- α . Additionally, IL-10 induces the secretion of IL-1Ra by macrophages, so blocking IL-1 effects (Maynard and Weaver 2008; Mosser and Zhang 2008). IL-10-deficient mice have been observed to be more susceptible to mycobacterial infection, confirming the importance of IL-10 for the regulation of immune responses against intracellular pathogens (Murray and Young 1999).

TGF- β is secreted in a latent form that requires proteolysis to become active. Plasmin is considered the most important enzyme involved in TGF- β activation, but thrombospondin-1, reactive oxygen species, and integrins show the same action (Khalil 1999; Taylor 2009). In general, TGF- β dampens the function of inflammatory cells and promotes that of Treg cells, which induce tolerance (Wan and Flavell 2007). It acts in a similarly to IL-10 but in a milder way (Chen and Wahl 1999; Opal and DePalo 2000). TGF- β shows contrasting effects on macrophages, depending on their activation status. TGF- β induces an initial pro-inflammatory response when acting on resting monocytes, but anti-inflammatory effects on activated macrophages (Ashcroft 1999), where it inhibits the secretion of TNF- α and IL-1. The immunosuppressive effect of TGF- β has been shown by the restoration of the capacity of peripheral blood monocytes from TB patients to secrete IFN- γ following TGF- β blocking (Hirsch, Hussain et al. 1996).

9. Mycobacterial infection of macrophages

9.1. Mycobacteria and TLRs

Mycobacteria have developed mechanisms to escape the immune response, yet they express PAMPs able to activate macrophage and dendritic cells via TLR2 and TLR4 agonism. TLR2 forms heterodimers with TLR1 and TLR6, which allows interactions with a broad spectrum of ligands. TLR2/TLR1 recognizes triacylated lipoproteins whereas TLR2/TLR6 recognizes diacylated lipoproteins and lipoteichoic acid (LTA)(Kumagai, Takeuchi et al. 2008). The mycobacterial lipoglycans phosphoinositol-capped lipoarabinomannan (LAM) and phosphatidyl-myoinositol mannosides and two lipoproteins, LpqH (19 kDa mycobacterial lipoprotein) and LprG, constitute the major mycobacterial ligands of TLR2. The role of TLR4 is not well characterized except for the fact that blocking TLR4 with antagonists eliminates *M. tuberculosis*-induced human alveolar macrophage apoptosis (Means, Jones et al. 2001; Gehring, Dobos et al. 2004; Krutzik and Modlin 2004). It is also known that, in contrast to *M. tuberculosis*, live *M. avium* and *M. bovis* BCG do not activate cells in a TLR4-dependent manner (Means, Wang et al. 1999; Wang, Lafuse et al. 2000; Heldwein, Liang et al. 2003). TLRs do not work as phagocytic receptors for the uptake of mycobacteria, like CR3, ManR and FcRs do. However, TLR activation promotes phagocytosis by inducing the expression of macrophage scavenger receptor-1 (MSR1), C-type lectins, CD36, complement and Fc receptors (Doyle, O'Connell et al. 2004; Mae, Iyori et al. 2007).

The recognition of mycobacterial LAM by macrophage TLR2 in a CD14-dependent way leads to the activation of a pro-inflammatory response (Savendra, Delude et al. 1996; Means, Lien et al. 1999). The ensuing signalling induces NF- κ B activation and secretion of IL-12, which in turn drives the secretion of IFN- γ (mainly by NK cells), TNF- α , IL-1 β and IL-6 (Zhang, Doerfler et al. 1993; Flynn and Chan 2001). In particular, ManLAM has been found to inhibit the secretion of the pro-inflammatory cytokine IL-12 and promote instead

the secretion of anti-inflammatory cytokines such as IL-10 and TGF- β that deactivate host cells, and IL-4, that directs the immune response to Th2 (Flynn and Chan 2001; van Crevel, Ottenhoff et al. 2002; Appelmelk, den Dunnen et al. 2008). This creates conditions for the intra-cellular persistence of mycobacteria.

TLR stimulation favours the formation of reactive oxygen and nitrogen species that play a key role in microorganism killing. In the case of mycobacteria, intracellular killing is nitric oxide- but not oxygen metabolite-dependent, and TLR activation results in an increase in NOS2 which provides a better capacity to generate NO and kill these microorganisms (Raupach and Kaufmann 2001). TLR2 activation is also responsible for an enhanced expression of the antimicrobial peptide cathelicidin that is active against mycobacteria (Sundaramurthy and Pieters 2007). Additionally, TLR2 agonism down-regulates the expression of MHCII by macrophages (Noss, Pai et al. 2001), weakening antigen presentation and T cell activation (Alvarez, Zwilling et al. 2003; Banaiee, Kincaid et al. 2006).

Concerning other TLRs, an attenuated TLR9 activation of human macrophages by *M. tuberculosis* DNA has been recently suggested to contribute to the insufficient response of these cells to virulent mycobacteria (Kierner, Senaratne et al. 2009).

9.2. Subversion of the immune response by mycobacteria

9.2.1. Defences against oxidative mechanisms

The survival of intracellular bacteria depends on how they manage to avoid the microbicidal mechanisms of macrophages. Oxygen and nitrogen reactive species constitute a powerful machinery to kill intra-cellular pathogens. Mycobacteria are not sensitive to oxygen but to nitrogen reactive species, but oxygen metabolism could also be of some relevance concerning mycobacterial growth control due to the interaction of the superoxide anion radical with NO that generates the powerful oxidant molecule peroxynitrite (Fig. 8). IFN- γ treated macrophages effectively kill mycobacteria by means of reactive nitrogen

intermediates, while ROS play a secondary role (Chan, Chan et al. 2001). Mycobacteria have developed mechanisms of resistance that are based on a detoxification machinery consisting of molecular scavengers, antioxidant enzymes and repair systems (Shiloh and Nathan 2000). Indeed, both *M. tuberculosis* and *M. avium* express superoxide dismutase and catalase activities that convert superoxide in O₂ and H₂O (Kusunose, Ichihara et al. 1976; Mayer and Falkinham 1986; Mayer 1986; Wayne and Diaz 1988; Manca, Paul et al. 1999). The contribution of these enzymes to the resistance to oxidative killing was demonstrated by Manca et al., who observed that *M. tuberculosis* strains expressing low levels of the catalase KatG are more sensitive to oxidative killing by macrophages (Manca, Paul et al. 1999).

9.2.2. Effect of mycobacteria on phagosome maturation

After being recognised by TLRs and/or internalised after ligation to phagocytic receptors on the macrophage surface, mycobacteria employ strategies to overcome the killing mechanisms of the host cell and create an environment favourable to their intracellular survival and replication. One of the main strategies is based on the arrest of phagosomal maturation. *M. tuberculosis* and *M. avium* can impair phagosomal acidification by inhibiting the trafficking of an ATP-dependent vacuolar proton pump (Schaible, Sturgill-Koszycki et al. 1998). Lack of acidification results in an inefficient or absent action of proteolytic/hydrolytic enzymes delivered to the phagosome by lysosomal fusion. Mycobacterial phagosomes do not mature properly. They retain the early endosomal marker Rab5 and transferrin receptor, and there is no acquisition of the late endosome markers Rab7 and LAMP1, nor of the tryptophan/aspartate-containing coat protein (TACO) which is a F-actin binding protein implicated in cytoskeletal organization and vesicle fusion (Figure 5) (Bhatt and Salgame 2007; Sundaramurthy and Pieters 2007). One of the mycobacterial molecules responsible for blocking phagosomal maturation is ManLAM, which inhibits increases in cytosolic calcium that are necessary for the recruitment of phosphatidylinositol-3-kinase (VPS34) to the phagosome by Rab5,

diminishes the formation of phosphatidyl-inositol-3-phosphate (PI3P) and its association with early endosomal antigen 1 (EEA1), finally blocking the recruitment of Rab7 that regulates the fusion between phagosomes and lysosomes (Kang, Azad et al. 2005; Shimada, Takimoto et al. 2006; Torrelles, Azad et al. 2006). The iron concentration in *M. avium* and *M. tuberculosis* phagosomes has been reported to be higher than that in phagosomes containing non-pathogenic *M. smegmatis* (Wagner, Maser et al. 2005), and this may be due to a mechanism not requiring energy employed by pathogenic strains for iron uptake (Ratledge 2004). The altered characteristics of mycobacterial phagosomes prevent the fusion of lysosomes and create better conditions for mycobacterial survival (Pieters 2001; Amer and Swanson 2002). The block in phagosome maturation does not occur if macrophages are activated by IFN- γ before infection, in which case mycobacteria can be more efficiently eliminated (Schaible, Sturgill-Koszycki et al. 1998; Hostetter, Steadham et al. 2002). The activity of IFN- γ is antagonized mostly by IL-10, a potent anti-inflammatory, de-activating cytokine secreted by mycobacterium-infected cells (Bermudez and Champs 1993). Mycobacterial phagosomes from IL-10 knockout mice have been reported to be more acid than those from normal mice (Via, Fratti et al. 1998).

9.2.3. Other effects of mycobacteria on macrophage function

After becoming established within macrophages, mycobacteria start to replicate and secrete proteins that, in addition to the mechanisms outlined in the preceding paragraph, help to down-regulate host cell responses. For instance, CFP-10 (10-kDa culture filtrate protein) is able to reduce NO production by macrophages even after IFN- γ activation, as well as to down-regulate the expression of the co-stimulatory molecule CD80 (Trajkovic, Natarajan et al. 2004). Studies on macrophage or monocyte infection with *M. tuberculosis* or *M. avium*, respectively, reported a reduction in co-stimulatory receptors and MHCII expression, and that they were less effective in presenting antigens and inducing T cell

activation and proliferation (Mohaghehpour, Gammon et al. 1997; Kan-Sutton, Jagannath et al. 2009). These effects compromise the development of an adaptive immune response.

10. Potential application of natural compounds in human disease

Nature is a constant source of new compounds with potential to be employed in human diseases. Numerous natural substances of different chemical structure, showing antimicrobial activity, have been identified as potentially useful (Saleem, Nazir et al. 2010). Plants are the origin of 25% of the drugs currently used (Demain 2009).

Since the discovery of penicillin by Alexander Fleming, many compounds have been isolated from microorganisms and plants and are still found in the market, e.g. cephalosporin, erythromycin, vancomycin, fusic acid (Pelaez 2006). Streptomycin, a protein synthesis inhibitor, is a classical example of successful anti-mycobacterial compound. Originally isolated from the actinobacterium *Streptomyces griseus* in 1943, it is still in use as first-line compound to treat *M. tuberculosis* infection.

Some natural compounds with antibiotic activity are already close to clinical trials as potential candidates for tuberculosis treatment, e.g. pleuromutilin, thiolactomycin and cerulenin. The last two are inhibitors of a fatty acid synthase, an important target in mycobacteria (de Souza 2009). Mascia Lopes et al observed that extracts from *Davilla elliptica* were anti-mycobacterial toward broth-grown organisms (Mascia Lopes, Polesi Placeres et al. 2007). These extracts modulated macrophage responses, increasing oxidative and nitrosative stress, as well as an augmenting TNF- α secretion. The long list of plant compounds reported to be active against different mycobacterial strains confirms the potential of this source (Newton, Lau et al. 2000). It is hoped that some of them could prove effective against resistant strains. Immunostimulation has been observed after treating macrophages with chlorophyllin. This chlorophyll derivative enhanced the phagocytosis of gram-negative bacteria and was effective in preventing cell apoptosis through an increase in the expression of anti-apoptotic genes (Sharma, Kumar et al. 2007).

Another plant derivative, the compound albacol, induced an anti-inflammatory effect suppressing the LPS-induced secretion (i.e. TLR4-dependent) of pro-inflammatory cytokines by macrophages and dendritic cells (Liu, Shu et al. 2008; Liu, Shu et al. 2008). Mushrooms are an important source of immunomodulatory molecules (Borchers, Krishnamurthy et al. 2008) such as davallialactone, that suppressed nitric oxide generation as well as the expression of co-stimulatory molecules, therefore compromising both innate and adaptive immunity (Lee, Lee et al. 2008).

β -glucans from fungi and some herbs stimulate hematopoiesis and have been used as adjuvants in cancer treatments since they potentiate the anti-tumour immune response. β -glucans are recognized by macrophages via several receptors, such as TLR2, dectin-1 and CR3. After being ingested, they are processed and released as small fragments. These small fragments are taken by phagocytes and presented to CD4⁺ T cells via MHCII. In this way β -glucans trigger an innate immune response, enhancing phagocytosis and secretion of pro-inflammatory cytokines and adaptive immune response through an increase in CD4⁺ cell proliferation (Novak and Vetvicka 2008; Chan, Chan et al. 2009).

Extracts from *Hippophae rhamnoides* have shown an antioxidant capacity similar to vitamin C (Geetha, Singh et al. 2005). Similar properties have also been observed for statins isolated from fungi (Benati, Ferro et al. 2010); quercetin, present in apples, berries and onion (Kumar, Sehgal et al. 2008; Woo, Kim et al. 2008); and resveratrol, present in grapes, berries and peanuts (Mikstacka, Rimando et al. 2010). Besides an anti-oxidant activity, these compounds have been reported to inhibit iNOS and NF- κ B activation (de la Lastra and Villegas 2007; Iyori, Kataoka et al. 2008; Youn, Lee et al. 2009), a potentially immunosuppressive effect due to a reduction in the secretion of pro-inflammatory cytokines.

Genetic polymorphisms can also be a cause of immunomodulation. Even a single point mutation in genes that express PRRs can change the protein and influence the immune response (Schroder and Schumann 2005). Polymorphisms can also limit directly

immune responses or regulate the availability of immunomodulating molecules. Like any other drug, immunomodulatory drugs are subjected to cellular clearance such as by the cytochrome P450 system expressed from CYP genes (Zhou 2008). Inter-individual variability in the expression of CYP genes results in different drug metabolic rates and elimination among individuals (van der Weide and Steijns 1999). Thus, the efficacy of immunomodulatory compounds is also dependent on the genetic background.

10.1. Quinones

Tabebuia avellanedae, also known as “Ipe-roxo” or lapacho, is an evergreen tree of the Bignoniaceae family that grows in many tropical/subtropical countries. Natives of South America have traditionally used this plant for medicinal purposes. The use of teas made with the bark of *Tabebuia* is currently registered as a dietary supplement by the Food and Drug Administration (Gomez Castellanos, Prieto et al. 2009).

Several compounds have been identified in the bark of *Tabebuia* plants, and among them some naphthoquinones raise a particular interest. Lapachol (NSC-11905) is a 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone and the most abundant quinone present in the Bignoniaceae family. It is structurally related to vitamin K (Petrova et al., 2000) of which it is an antagonist (Preusch and Suttie, 1984).

Other 1,4-naphthoquinones such as β -lapachone, juglone, plumbagin, menadione, atovaquone and lawsone (Fig. 9) have had an application in clinics. Atovaquone is used for the treatment of *Pneumocystis carinii* pneumonia, and is used in association with proguanil as a well tolerated anti-malarial compound (Taylor and White 2004; Nakato, Vivancos et al. 2007). Plumbagin and β -lapachone have been reported to have anti-tumour activity (Boothman, Trask et al. 1989). The anti-tumour activity of plumbagin has been ascribed to NF- κ B inhibition (Sandur, Ichikawa et al. 2006). The same mechanism of action was responsible for the immunosuppressive effect of plumbagin on T cells (Checker, Sharma et

al. 2009). Plumbagin has also been reported to be anti-mycobacterial (Mossa, El-Feraly et al. 2004).

Menadione, another naphthoquinone, is a vitamin K analog that generates oxidative stress, which is a common characteristic of this group of substances (Castro, Mariani et al. 2008). However, menadione did not cause an increase in levels of superoxide dismutase of mycobacteria (Garbe, Hibler et al. 1996). Juglone has been reported to be anti-proliferative and cytotoxic to tumor cells (Bonifazi, Rios-Luci et al. 2010; Montenegro, Araujo et al. 2010). Lawsone is present in henna dyes used for tattooing. It stimulates oxidative stress in keratinocytes, but weakly compared with other compounds of this group (Klaus, Hartmann et al. 2010).

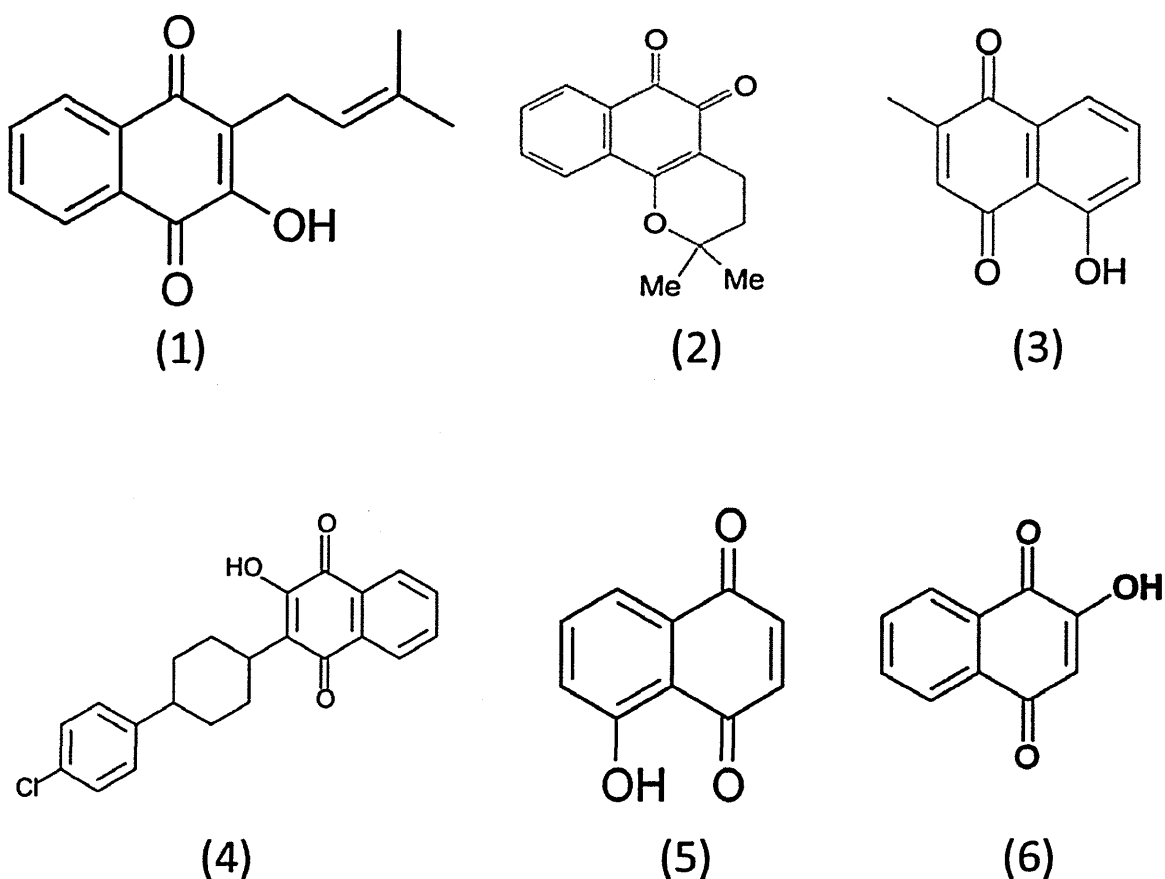


FIGURE 9. Chemical structure of 1,4-naphthoquinones. 1, Lapachol; 2, β -lapachone; 3, plumbagin; 4, atovaquone; 5, juglone; 6, menadione.

Quinones in general and hydroxy-naphthoquinones in particular have been reported to be substrates for membrane-bound bacterial anaerobic reductases (Rothery, Chatterjee et al. 1998). They can also act as substrates for the cytochrome bc system of prokaryotes and eukaryotes (Kessl, Moskalev et al. 2007), and promote the generation of reactive oxygen species at mitochondrial level (Docampo, De Souza et al. 1978; Bolton, Trush et al. 2000; Goulart, Falkowski et al. 2003). This occurs by NADPH-cytochrome P450 reductase-catalysed reduction, which generates semiquinone radicals. Semiquinones can be reconverted into the original quinones by reducing oxygen to superoxide (O_2^-), which gives rise to ROS (Fig. 10) (Bachur, Gordon et al. 1979; Kumagai 1997; Goulart, Falkowski et al. 2003).

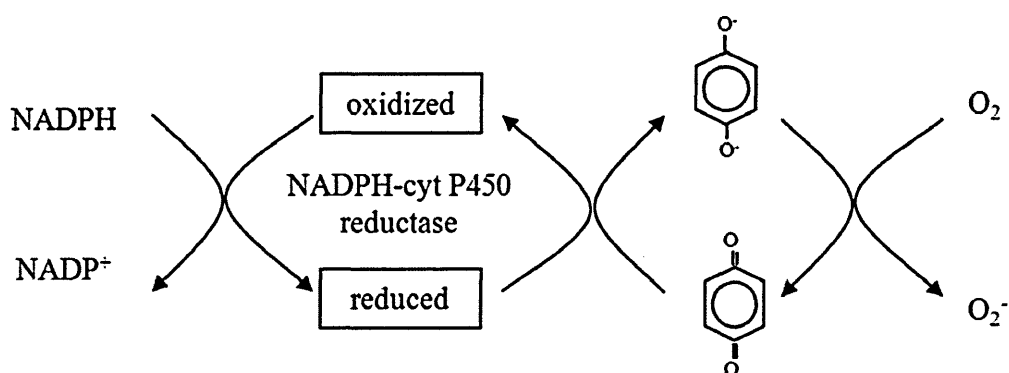


FIGURE 10. Naphthoquinone reduction by NADPH-cytochrome P450 reductase. Redox cycling activity can render naphthoquinones toxic. They are reduced by cytochrome P450 reductase at the expense of NADPH, generating semi-naphthoquinone and superoxide anion radicals. Superoxide anions are reactive, oxidizing molecules that can modify proteins, DNA and lipids. Extracted from Bachur et al (Bachur, Gordon et al. 1979), with modifications.

Lapachol was the 1,4-naphthoquinone extensively studied during the present work. It is a multi-active compound reported to have larvicidal (Oliveira, Lemos et al. 2002; Lemos, Monte et al. 2007), antiviral (Sacau, Estevez-Braun et al. 2003), bactericidal (Guiraud, Steiman et al. 1994; Oliveira, Miranda et al. 2001; Machado, Pinto et al. 2003; Park, Lee et al. 2006; Pereira, Machado Tde et al. 2006), fungicidal (Guiraud, Steiman et al. 1994; Breger, Fuchs et al. 2007), anti-protozoal (Duarte, Dolabela et al. 2000; De

Moura, Emerya et al. 2001; Teixeira, de Almeida et al. 2001; de Andrade-Neto, Goulart et al. 2004; Lima, Correia et al. 2004; Perez-Sacau, Estevez-Braun et al. 2005; Salas, Tapia et al. 2008), anti-inflammatory (de Almeida, da Silva Filho et al. 1990; Bezerra, De Oliveira et al. 2005; Lira, Sester et al. 2008) and limited anti-tumor (Rao, McBride et al. 1968; Oliveira, Lemos et al. 2002; Ravelo, Estevez-Braun et al. 2004; Bezerra, De Oliveira et al. 2005) activities, with different efficacies. Apart from its antifungal activity (Guiraud, Steiman et al. 1994), the antimicrobial concentrations of lapachol were found to be > 30µg/mL. Lapachol has been reported to be anti-proliferative at high concentrations (Duarte, Dolabela et al. 2000; Oliveira, Lemos et al. 2002; Bezerra, De Oliveira et al. 2005; Esteves-Souza, Figueiredo et al. 2007), whereas some reports describe an anti-metastatic effect *in vivo*, at non-toxic doses (Balassiano, De Paulo et al. 2005; Maeda, Murakami et al. 2008). Based on *in vitro* assays, lapachol has been suggested to be anti-proliferative because it inhibits DNA-topoisomerase II- α , although only a partial inhibition was achieved at 0.20 mM (Esteves-Souza, Figueiredo et al. 2007). Knowledge on the clinical use of lapachol as an anti-infectious agent is lacking. Within the wide spectrum of activities of lapachol, no anti-mycobacterial action has been reported to date. No *in vitro* or *in vivo* studies are available either about its influence on innate immunity responses, i.e. on whether it shows immunomodulatory characteristics.

In vivo experiments have shown that the oral administration of lapachol (100 mg/kg) to rats for 5 days did not produce any alteration in total body nor in organ weights (de Cassia da Silveira and de Oliveira Guerra 2007). Only a reduction in male seminal vesicle weight was observed, while gamete production was not affected. When lapachol was administered at the same dose to pregnant rats, there were no indications of maternal toxicity or of toxicity toward pre-implantation embryo development. The only negative effect was a higher proportion of reabsorptions and a lower weight of fetuses and placentae (de Almeida ER 1988; Maganha J 2006). During studies on the anti-tumor activity of lapachol in rats, a maximum tolerated and a minimum effective oral dose of 750 and 90

mg/kg, respectively, were observed (Rao, McBride et al. 1968). Again, there was relatively little effect on body weight. The most extensive toxicology studies were carried out by Morrison et al. (Morrison, Brown et al. 1970). They reported high values for single dose LD50s in mice and rats: 0.6 g/kg and 2.4 g/kg, respectively. No lethality was found by administration of up to 2 g/kg daily for a month to dogs. Gross and anatomical findings were not remarkable: all tissues, including bone marrow and kidneys, were normal. There was only some hemosiderin deposition in liver Kupffer cells and splenic red pulp, and reversible anemia was observed. In monkeys, lethal doses were of 0.5-1.0 g/kg over 5-6 days. Anemia was again the major feature, which returned to normal during the period of drug administration. Bilirubinuria and proteinuria were also detected.

Human studies of lapachol were performed by Block et al. (Block, Serpick et al. 1974) in relationship with its presumed anti-tumour activity, based on the animal studies mentioned in the paragraph above. Lapachol, although without anti-tumour activity, was well tolerated at ≤ 1.5 g/day for up to at least 21 days (21 mg/kg for a 70 kg body weight). At ≥ 2 g/day (29 mg/kg for a 70 kg body weight) nausea, vomiting and an anti-coagulant effect were observed (Block, Serpick et al. 1974; Sieber, Mead et al. 1976). The latter probably derives from its being a vitamin K antagonist, since vitamin K can correct the effect. These toxic effects, observed with high doses, were not severe and were reversible. No hematologic, renal or liver function impairments were detected after the lapachol treatments. The serum concentrations of lapachol achieved ranged from 6 to 26 mg/L and peaked at 15-26 mg/L, 8 hours after a single oral administration of 35 mg/kg. A convenient feature was that serum levels were rather persistent, and after 24 hours the concentration of lapachol was still 30-50% of the peak value. Persistence was improved by administering 3 smaller doses in consecutive days (Block, Serpick et al. 1974).

10.2. Other compounds causing microbial growth arrest used during the present work

Other synthetic compounds employed in this study were (Figure 11): 1,2-*O*-diacyl-3-*O*-glucosylglycerol (9A), a chemically modified glycolipid of plant origin previously reported be active against Gram-positive and Gram-negative bacteria and *Mycobacterium tuberculosis* (Cateni, Bonivento et al. 2007); N¹-(3-aryl-1-(pyridin-2-,3-,4-il)-3-oxo)-propyl)-2-pyridine carboxamidrazone, a synthetic compound active against against *M. tuberculosis* and *M. avium* (Mamolo, Falagiani et al. 1999; Banfi, Mamolo et al. 2001) ; and the compounds AC178, AC201, AC202, AC203, and AC204 of unknown chemical structure due to the manufacturers' protection policy.

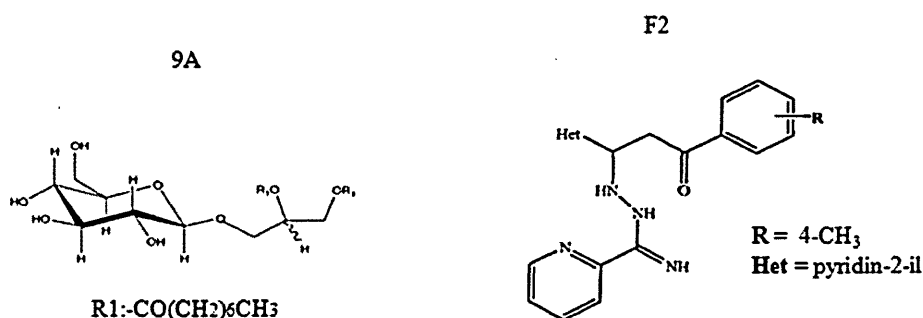


FIGURE 11. Chemical structure of the compounds 9A and F2 used in this study. 1,2-*O*-diacyl-3-*O*-glucosylglycerol (9A) (Cateni, Bonivento et al. 2007) and N¹-(3-(4-CH₃)aryl-1-(pyridin-2-il)-3-oxo)-propyl)-2-pyridine-carboxamidrazone (F2) (Banfi, Mamolo et al. 2001).

11. Rationale and strategy of the present work

In order to explore the potential of novel compounds as possible therapeutic drugs it is appropriate to study their effect on mammalian cell functions, at molecular level. In this direction, the use of cell culture systems can provide valuable information before engaging in *in vivo* experimentation. Cell cultures allow the study of both resting and activated cells, providing the possibility to mimic physiological activations or situations. The information obtained can give hints about how the situation could be when the drug is administered to a complex organism, and work as a first filter to exclude a number of candidate drugs at an

early stage, avoiding unnecessary animal experimentation. Drugs that pass the cell culture stage of the evaluation will obviously need to be subjected to *in vivo* studies, and clinical trials will eventually follow.

During the present work, the anti-mycobacterial effect of candidate compounds was evaluated, first on bacteria growing in liquid broth and subsequently on intracellular bacteria, using macrophage-like cells as host cells. The next stage was to examine the influence of candidate compounds on activation-inducible macrophage functions and protein expression, to gain knowledge on their immunomodulating and functional effects.

The methodology for the evaluation of anti-mycobacterial compounds included:

1. Determination of their capacity to arrest the growth of *M. avium* in liquid broth.
2. Assessment of their effect on macrophage-like cells viability.
3. Determination of their capacity to arrest the intra-macrophage growth of *M. avium*.
4. Study of their effects on macrophage responses to activation by TLR2 agonism or IFN- γ , or to *M. avium* infection, such as: oxidative and nitrosative stress, phagocytosis, antigen presentation and cytokine secretion.
5. Analysis of the changes in the protein expression pattern of activated or infected macrophages elicited by the compound studied.

MATERIALS AND METHODS

1. Compounds of potential antibiotic activity

Lapachol (98.8% pure, according to HPLC), was kindly provided by the Antibiotic Department of the Federal University of Pernambuco (Brazil). The providers ensure the drug is LPS-free. It is of plant origin and is currently employed in human cancer treatment, for which it must be LPS-free. Concentrated stock solutions were made in DMSO. Clarithromycin (VECLAM®, Abbott), ethambutol (ETAPIAM®, PIAM), and rifampicin (RIFADIN®, Aventis) were kindly provided by Dr. Roberto Luzzati (Department of Infectious Diseases, University Hospital, Trieste, Italy). Concentrated stock solutions were made in phosphate-buffered saline (PBS). The compounds N1-(3-(4-CH₃)aryl-1-(pyridin-2-yl)-3-oxo)-propyl)-2-pyridine-carboxamidrazone (F2) and 1,2-*O*-diacyl-3-*O*-glucosylglycerol (9A) were provided by Dr. Elena Banfi (Department of Biomedical Sciences, Microbiology Section, University of Trieste, Italy). Concentrated stock solutions were prepared in DMSO. The AC series compounds (AC 178, 201, 202, 203 & 204) were kindly provided by Avidin Ltd (Hungary). Concentrated stock solutions were made in DMSO. The final concentration of DMSO in mammalian cell cultures was always ≤1%, a concentration known to show no effects. Controls were always run containing the same concentration of DMSO as that introduced with the addition of the different compounds to cultures.

2. Main solutions

Protease inhibitor stock solutions (Complete®, Roche Diagnostics, Mannheim, Germany) were prepared by dissolving one tablet in 2 mL of water, yielding a 25x solution.

FACS buffer contained 5mM glucose, 0.1% (w/v) NaN₃ and 1% (v/v) fetal calf serum (FCS), in PBS.

5X SDS-PAGE running buffer was composed of 30g of TRIS base, 144g of glycine and 5g of sodium dodecyl sulphate (SDS) per litre. Western blotting solutions were made of 0,5X running buffer plus 5% (v/v) methanol.

The isoelectrofocusing strip equilibration buffer used for the proteomic studies contained 50mM Tris HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and trace amounts of bromophenol blue (BPB).

5X TBE buffer consisted of 54 g of TRIS base, 27.5g boric acid and 20 mL EDTA (0.5M, pH 8.0) per litre.

3. Cell cultures

3.1. Mammalian cell cultures

THP-1 human monocytic cells (Auwerx 1991) (ATCC TIB-202, American Type Culture Collection, Rockville, MA) were cultured in a humidified, 5% CO₂ incubator, at 37°C, in RPMI 1640-GlutaMAX™ medium (Gibco®, Invitrogen, Milano, Italy) containing 25 mM glucose, 10 mM Hepes, 1 mM sodium pyruvate, and 10% (v/v) fetal bovine serum (EuroClone®, Celbio, Milano, Italy). The culture medium used for cell sub-culturing and amplification contained additionally 100 U/mL penicillin, 0.1 mg/mL (170 µM) streptomycin and 0.25 µg/mL (270 nM) amphotericin B (Sigma-Aldrich). Cells were continuously treated with 20 nM phorbol myristate acetate (PMA; Sigma). After four days, differentiation was complete and stimulations or infection were started, always in the presence of 20nM PMA.

3.2. *Mycobacterium avium* cultures

M. avium strain 485 (serovar 21, transparent colonies) was kindly provided by the Istituto Superiore della Sanita' (Rome, Italy) (Fattorini, Xiao et al. 1994). It was originally isolated from the blood of an AIDS patient. The use of *M. avium* was based on: 1) the increase of infections caused by this non-tuberculous mycobacterium, and 2) the

similarities between *M. tuberculosis* and *M. avium* (Sturgill-Koszycki, Schlesinger et al. 1994; Schaible, Sturgill-Koszycki et al. 1998) in their intracellular behaviour, which make *M. avium* a good model of *M. tuberculosis* for *in vitro* studies.

3.2.1. Growth on agar

Bacterial dilutions were plated on Middlebrook 7H11 agar supplemented with 10% oleic acid-albumin-dextrose complex (OADC, Difco Laboratories, Detroit, MI) and grown at 37°C in microaerophilic jars until discrete colonies (colony forming units: CFU) were observed, usually after 10 to 15 days of incubation.

3.2.2. Growth in liquid medium

M. avium was grown at 37°C in Middlebrook 7H9 broth supplemented with 10% albumin-dextrose complex (ADC, Difco Laboratories, Detroit, MI) and 0.5% (v/v) glycerol until approximately 10^9 cells/mL. The exact number of viable colony counts was determined by growth on Middlebrook 7H11 agar plates. Bacterial suspensions were stored at -80°C.

3.2.3. Susceptibility of *M. avium* to potentially antibiotic compounds (growth in liquid culture)

This was performed by the microplate Alamar Blue assay (Franzblau, Witzig et al. 1998), using 96-well plates (Falcon 3595; Becton Dickinson, Lincoln Park, NJ). The method is based on the change of colour from blue to pink on bacterial growth, indicative of the reduction of resazurin, to resorufin, Serial dilutions of the compound under study were done so that 10 out of the 12 wells in each column contained 100 µl of 7H9 broth and the test compound at concentrations halving sequentially from one well to the next in each column, from a maximum of 128 down to 0.5 µg/mL. Eight control wells, in a row, contained only 7H9 broth and no lapachol. Subsequently, 0.1 mL of a *M. avium* inoculum containing 10^6 bacteria was added to each of the wells, the plate was sealed with Parafilm and initially incubated at 37°C for 5 days. To follow bacterial growth, 50 µL of Alamar Blue mix, consisting of CellTiter-Blue™ reagent (Promega, Madison, WI) and 10% (v/v)

Tween 80 (1:1), was added in the first place to one of the control wells. If the colour did not turn pink after 24 hours at 37°C, Alamar Blue mix was added to another of the control wells and the colour checked after further 24 hours. This was repeated until the pink colour of a control well indicated good *M. avium* growth. At that point, Alamar Blue was added to all the wells in the plate, i.e. those corresponding to all the different concentrations of the test compound as well as the remaining control wells. After 24 hours at 37°C, an image showing the colour of the well liquids was recorded (see figure below). The minimal inhibitory concentrations (MIC) of the test compound correspond to its concentration in the first blue well in the column (indicating no growth) immediately after a series of pink-coloured wells (indicating bacterial proliferation).

3.2.4. Bacteriostasis against bacterial killing

To determine whether lapachol and AC201 were bacteriostatic or bactericidal, *M. avium* (starting concentration 10^7 /mL) was cultured in the absence or presence of 16 µg/mL (66µM) or 32 µg/mL (132µM) lapachol, or 32 µg/mL (800µM) AC201, in Falcon® tubes. Aliquots were taken from the cultures at day=0, 1, 3 and 7, diluted appropriately and plated on agar as described above in section 3.2.1. CFUs were counted after 10 to 15 days of incubation in microaerophilic jars, at 37°C.

3.2.5. Intracellular proliferation of *M. avium*

THP-1 macrophage-like cells were infected with *M. avium* for 4 hours in wells containing 10^6 cells each, at a bacterium-to-cell ratio of 30:1 (Pittis, Muzzolin et al. 2003), then washed 3 times with PBS and kept in culture at 37°C in a 5% CO₂ incubator. The number of cell-associated bacteria was determined at time zero (end of internalisation period), day 3 and day 7. Cells were scraped and lysed with 0.025% SDS, after which the SDS in the lysates was neutralized with 0.5% albumin (Bermudez, Petrofsky et al. 2004). Lysates were then diluted and plated on Middlebrook 7H11 agar and incubated for 10 to 15 days as described above in 3.2.1, in order to determine *M. avium* CFUs. Some of the colonies could have originated in extra-cellular bacteria that survived attached to the

plastic surface of wells, not having been washed off at the end of the phagocytosis period. These hypothetical, non- or slowly proliferating extra-cellular bacteria would in fact contribute more to the total number of CFUs at early times ($t=0$ and day 3) than at day 7, when intra-cellular growth is under way. Therefore, the intra-cellular growth data corresponding to day 7 are likely to be underestimated relatively to the CFUs determined at $t=0$.

3.2.5.1. Intracellular *M. avium* growth arrest by test compounds

The procedure was as indicated above (3.2.5) for intracellular proliferation, but adding 16 $\mu\text{g/mL}$ (66 μM) or 32 $\mu\text{g/mL}$ (132 μM) lapachol; 32 $\mu\text{g/mL}$ (800 μM) AC201; 0.56 $\mu\text{g/mL}$ (750nM) clarithromycin; 1.6 $\mu\text{g/mL}$ (1.9 μM) rifampicine or 0.35 $\mu\text{g/mL}$ (1.71 μM) ethambutol at time zero (end of internalisation period). The number of cell-associated viable bacteria was also determined at day 3 and day 7, as described above (3.2.5).

3.3. *Staphylococcus aureus* and *Salmonella typhimurium* cultures

S. aureus (502A strain) and *S. typhimurium* (SL3261AT, GFP-expressing) were used as examples of Gram-positive and Gram-negative organisms, respectively. They were grown in Luria broth (LB) overnight at 37°C and the number of viable bacteria was determined by plating on Luria agar and incubation overnight at 37°C. Bacteria were stored at -80°C.

3.3.1. Susceptibility of *S. aureus* and *S. typhimurium* to test compounds (growth in liquid culture)

This was performed according to the methodology described by Andrews, J. M. (Andrews 2001). The MIC of lapachol and AC201 toward *S. aureus* and *S. typhimurium* was determined in tubes containing 5 mL of LB broth and serial dilutions of the compounds being tested, from 256 to 0.5 $\mu\text{g/mL}$. Bacteria were then inoculated at the initial concentration of 10^5 CFU/mL. Positive controls did not contain any test compound, and negative controls consisted of LB without both test compounds and bacteria. The tubes were incubated overnight at 37°C and growth was checked the next morning by turbidity

development. The MIC was the lowest concentration of the compound that prevented bacterial growth.

4. Cell morphology

THP-1 morphology was examined after treating the cells with the test compounds at their MICs for four days. Light microscopy photographs were taken using an inverted microscope (Nikon, TMS-F), at 100X magnification. Quantification of cell size was performed recording the pixels of 100 cell areas in each case. Forward and side light scatter were used to quantify cell size and granularity, respectively. Results are expressed as averages \pm SD and were analysed by ANOVA.

5. Apoptosis and necrosis

Macrophage apoptosis was quantified by detection of phosphatidylserine externalisation to the outer leaflet of plasma membranes, by Annexin V-FITC staining, according to the manufacturer's instructions (APOPTEST™-FITC, Nexins Research, Kattendijke, The Netherlands). Briefly, cells (10^5 to 10^6 /mL) were washed, resuspended with 0.49 mL of ice-cold binding buffer and added 5 μ L of 25 μ g/mL Annexin V-FITC. After 10 min incubation in the dark, at 4°C, cells were washed with 0.25 mL of binding buffer and analysed immediately by flow cytometry. Blanks were cells incubated with binding buffer alone, negative controls were cells in the absence of antibiotics and positive controls were cells incubated for 48 hours with 2 μ g/mL (3.4 μ M) etoposide in binding buffer, at 37°C.

Necrosis was evaluated by analyzing DNA fragmentation. DNA was prepared by standard phenol-chloroform extraction, after proteinase K digestion, from freshly harvested cells that had been treated or not with 32 μ g/mL (132 μ M) lapachol for 4 or 7 days or with 32 μ g/mL (800 μ M) AC201 for 4 days. DNA (200 ng/lane) was run on 0.8% agarose gels containing ethidium bromide.

6. Binding and internalisation of particles and bacteria

6.1. Latex beads

Plate-adherent macrophage-like cells were treated or not with P₃CSK₄ (300 nM) for 4 days and subsequently incubated with or without lapachol (132µM) for 4 additional days. Latex beads were then added (amine-modified, fluorescent yellow-green, Sigma-Aldrich, St. Louis, MO) at a 5:1 multiplicity (chosen on the basis of preliminary experiments), centrifuged for 15 min at 800Xg and left for 30 min at 37°C. After washing 3 times with PBS, cells were scraped off, resuspended in FACS buffer and analysed by flow cytometry (10⁴ cells per event). Phagocytosis (%) is expressed as [number of cells taking up any number of particles]/[total gated cell number] X 100. Phagocytic activity indicates the number of latex beads internalized into cells, and is expressed as the median fluorescence intensity (MFI) of the FACS histograms.

6.2. *S.aureus*

Plate-adherent cells were treated or not with P₃CSK₄ (300 nM) for 4 days and subsequently with lapachol (132µM) for 4 additional days. *S. aureus* were then added at a 50:1 bacteria/cell multiplicity. This ratio was chosen according to preliminary experiments. The plates were centrifuged for 15 min at 800Xg and incubated at 4 or 37°C for 30 min. Cells were then washed 3 times with PBS, scraped off and pelleted. Pellets were lysed with 0.2 mL of 1 mM NaOH (pH 11) and the lysates were diluted and plated in LB agar. After overnight incubation at 37°C, CFUs were counted. Results correspond to cell-associated CFUs.

6.3. *S. typhimurium*

Plate-adherent macrophages were treated or not with P₃CSK₄ (300 nM) for 4 days and subsequently with lapachol (132µM) for 4 additional days. Fluorescent (GFP) *S. typhimurium* were then added at a ratio of 50 bacteria/cell. This ratio was chosen according to preliminary experiments. The plates were centrifuged for 15 min at 800Xg

and incubated at 4 or 37°C for 30 min. Cells were then washed 3 times with PBS, scraped off and pelleted. Pellets were resuspended in FACS buffer (PBS containing 5 mM glucose, 0.1% NaN₃ and 1% FCS) and analysed by flow cytometry (10⁴ cells per event). Phagocytosis (%) is expressed as [number of cells taking up any number of particles]/[total gated cell number] X 100. Phagocytic activity indicates the number of bacteria internalized into cells, and is expressed as the median fluorescence intensity (MFI) of the FACS histograms.

6.4. *M. avium*

6.4.1. Preparation of *M. avium*-FITC

M. avium (3X10⁸ CFUs) were incubated with fluorescein isothiocyanate isomer 1 (FITC, Sigma-Aldrich, St. Louis, MO) for 30 min, at 20°C, in 1 mL of 50 mM Na₂CO₃-100 mM NaCl (pH 9.2) (de Boer, Bevers et al. 1996) and washed with PBS till the supernatants were no longer fluorescent. These labeled bacteria were resuspended in THP-1 cell culture medium and used to infect adherent THP-1 cells as described below.

6.4.1.1. Binding and internalisation by flow cytometry

Adherent cells were treated or not with P₃CSK₄ (300 nM) for 4 days and subsequently treated with lapachol (132μM) for 4 additional days. *M. avium*-FITC was then added at 30:1 multiplicity. This ratio was chosen according to previous work (Pittis, Muzzolin et al. 2003). After centrifugation for 15 min at 800Xg, they were left for 4 hours at 4 or 37°C and then washed 3 times with PBS. Cells were scraped off, resuspended in FACS buffer and analysed by flow cytometry (10⁴ cells per event). Phagocytosis (%) is expressed as [number of cells taking up any number of particles]/[total gated cell number] X 100. Phagocytic activity indicates the number of bacteria internalized into cells, and is expressed as the median fluorescence intensity (MFI) of the FACS histograms.

6.4.1.2. By fluorescent microscopy

Adherent cells were grown on round coverslips, treated with P₃CSK₄ (300 nM) for 4 days and then with lapachol (132μM) for 4 additional days. They were infected at 37°C

with *M. avium*-FITC at 30:1 multiplicity. This ratio was chosen according to previous work (Pittis, Muzzolin et al. 2003). After centrifugation for 15 min at 800Xg cells were left for 4 hours and washed with PBS. They were then fixed with 4% (w/v) paraformaldehyde (PFA) for 20 min, washed with PBS and permeabilized with 0.1% Triton X-100 (in PBS), for 5 min. They were washed, incubated with 0.1M glycine in PBS to block the PFA, washed again and counter-stained for actin by incubation with 0.4 U/mL Rhodamine Phalloidin (Invitrogen, OR), for 30 min, at RT, in the dark. The coverslips were washed, dried, mounted on slides using Vectashield (Vector Labs., Burlingame, CA) and sealed for further examination under the fluorescence microscope. Digital images were acquired and processed using Adobe Photoshop, version 6.0 (Mountain View, CA). Phagocytosis was quantitated by counting the number of fluorescent *M. avium* in 200 cells for each situation or condition. It is expressed as the ratio between the total number of bacteria ingested and the total number of cells.

7. Cell surface markers by flow cytometry

Adherent THP-1 macrophage-like cells were treated with P₃CSK₄ (300 nM) or IFN- γ (100 U/mL) for 4 days, then with lapachol (132 μ M) for 4 additional days. Cells were scraped off, pelleted, resuspended with 200 μ L of FACS buffer (PBS containing 5 mM glucose, 0.1% NaN₃ and 1% FCS) and incubated for 30 min at 4°C with 5 μ g/mL mouse IgG in order to block Fc receptors. The cells were washed twice with FACS buffer and then incubated with the corresponding antibody at 10 μ g/mL for 40 min, at 4°C. The following mouse monoclonal antibodies were used: for IFN- γ receptor, mAb sc-12755 (Santa Cruz Biotechnology, Santa Cruz, CA); for ICAM-1 (CD54), a mAb labeled with phycoerythrin (PE) (sc-107, Santa Cruz Biotechnology); for MHCII, a PE-labeled mAb (120P, Chemicon International, Temecula, CA); for CD16, mAb sc-20052 (Santa Cruz Biotechnology, Santa Cruz, CA); for CD32, mAb sc-13527 (Santa Cruz Biotechnology, Santa Cruz, CA); for CD64, mAb sc-1184 (Santa Cruz Biotechnology, Santa Cruz, CA);

for CD14, mAb sc-1182 (Santa Cruz Biotechnology, Santa Cruz, CA); for CD83, mAb sc-19678 (Santa Cruz Biotechnology, Santa Cruz, CA); for CD86, mAb sc-19617 (Santa Cruz Biotechnology, Santa Cruz, CA); for CD80, mAb sc-20077 (Santa Cruz Biotechnology, Santa Cruz, CA); for CD18, mAb sc-8420 (Santa Cruz Biotechnology, Santa Cruz, CA); for Mannose Receptor, mAb ab8918 (Abcam, Cambridge, UK); for TLR2, mAb TL2.1 (Alexis, San Diego, CA); for CD87, mAb sc-13522 (Santa Cruz Biotechnology, Santa Cruz, CA); and for SR-A, rabbit polyclonal Ab sc-20441 (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then washed three times with FACS buffer and, in the case of conjugated first antibodies, analysed directly by FACS. In the other cases, cells were resuspended with 0.1 mL of FACS buffer containing RPE-conjugated F(ab')₂ rabbit anti-mouse or FITC-conjugated polyclonal rabbit anti-goat or FITC-conjugated polyclonal swine anti-rabbit, incubated at 4°C for 40 min, washed and resuspended with 0.3 mL of FACS buffer for analysis. Flow cytometry (10⁴ to 3X10⁴ cells per event) was performed in a FACScalibur (Becton Dickinson, San Jose, CA). Results were analysed by means of the Cell Quest Pro software (Becton Dickinson). Data corresponding to 10⁴ or 3X10⁴ cells were recorded and subsequently gated within the appropriate forward- and side-light scattering area, to exclude non-viable cells from the analysis. Statistical analysis was performed on the medians from histograms of single parameters (FSC, SSC, FL-1, FL-2).

8. Cytokine secretion

The concentration of human cytokines in cell-free culture supernatants was determined using ELISA kits for TNF- α (Endogen, Pierce Biotechnology Inc., IL, USA), IL-10, IL-12 and IL-1 β (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions.

9. Subcellular fractionation

Cells were gently scraped off cell culture flask surfaces and the pellets washed successively with PBS and 9% sucrose-10 mM PIPES (pH 7.2), at 0–4°C. Cells were resuspended with 1 mL of 9% sucrose-10 mM PIPES (pH 7.2) containing protease inhibitors (Complete™, Roche Diagnostics GmbH, Mannheim, Germany) and passed repeatedly through a 23G needle until 95–100% disruption was achieved, according to observation by light microscopy. Nuclei and unbroken cells were pelleted at 600Xg (r_{\max}) and the nuclei-free supernatants (cytoplasms) were centrifuged at 230,000Xg for 1 hr, at 4°C. High speed supernatants (cytosolic fractions) were kept at –80°C, and high speed pellets (membrane fractions = plasma membrane + cytoplasmic organelles) were washed with 9% sucrose-10 mM PIPES (pH 7.2) containing protease inhibitors, resuspended with 0.2 to 0.4 mL of the same solution and kept at –80°C. Protein concentrations were determined by the method of Bradford (BioRad GmbH, München, Germany).

10. Protein Electrophoresis and Immunoblotting

Proteins of interest were detected by Western blotting of cytosolic or membrane cell fractions or total cell extracts. Electrophoreses were performed in 10, 12 or 14% SDS-polyacrylamide gels, loading 100 µg of protein per lane. Western blot membranes were probed with antibodies against gp91^{phox} sc-20782 (rabbit polyclonal, Santa Cruz, CA, USA), p47^{phox} sc-17844 (mouse mAb, Santa Cruz), CuZn-superoxide dismutase sc-11407 (rabbit polyclonal, Santa Cruz), Mn-superoxide dismutase (rabbit polyclonal, Upstate Biotech., NY, USA), grp78 (mouse anti-KDEL Ig, Stressgen, BC, Canada), β-Actin sc-47778 (mouse mAb, Santa Cruz, CA, USA), arginase ab13972 (mouse mAb, Abcam, Cambridge, UK), iNOS ab15323 (rabbit polyclonal Ab, Abcam, Cambridge, UK) or glyceraldehyde-3-phosphate dehydrogenase sc-25778 (GADPH, rabbit polyclonal, Santa Cruz), and subsequently with the appropriate alkaline phosphatase-conjugated secondary

antibodies. Bands were detected by developing with solubilized NBT-BCIP tablets (Roche Diagnostics GmbH, Mannheim, Germany).

11. Proteomics

11.1. Protein isolation and solubilisation

After having determined protein concentrations by the method of Bradford, cellular fractions containing 300 µg of protein were precipitated with 10% (final concentration, w/v) trichloroacetic acid (TCA), overnight, at 4 °C, and centrifuged at 10X000g for 15 min. Protein pellets were washed 4 times with 400µL of cold diethylether-ethanol (1:1, v/v), centrifuging each time at 10X000g for 10 min. Pellets were dried in the air for 10 min and solubilised in 240µL of isoelectrofocusing (IEF) rehydration solution containing 0.6% IPG buffer pH 3-11 NL (GE Healthcare Bio-sciences AB, Uppsala, Sweden). These samples were stored at -20°C until used.

11.2. Bidimensional separations (IEF/SDS-PAGE)

Proteins were initially separated according to their isoelectric point, on non-linear immobilized pH gradient strips (Immobiline™ DryStrip, pH 3-11 NL) of 13 cm (GE Healthcare Bio-sciences AB, Uppsala, Sweden). Sample loading was performed by in-gel re-swelling for 14 h, at RT. Isoelectric focusing was performed on an EttanIPGphorR IEF unit (Amersham Biosciences), at 20°C, using the following program: 50 V for 4 h, 500 V for 1 h, 1000 V for 2 h, and 8000 V up to 48 000 Vxh.

After electrofocusing, the strips were equilibrated with 2.5 mL of equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris-HCl pH 8.8, 2% [w/v] SDS, 25 mg DTT and bromophenol blue) for 10 min, at RT. This solution was then removed and a second equilibration was performed again for 10 min, with the same equilibration buffer but containing 62.5 mg of iodoacetamide instead of DTT. The second dimension of protein separation according to molecular mass was subsequently performed by placing each strip

at the top of 14% SDS-polyacrylamide gels ($180 \times 200 \times 1.5$ mm) that were then run at 12 mA per gel overnight, at RT.

11.3. Gel staining. Calculation of pI/s and molecular masses

After electrophoresis, gels were fixed for 1 hour in 10% (v/v) ethanol-7% (v/v) acetic acid, with shaking, and then stained for 48 hours with 0.12% Colloidal G-25 Coomassie Blue (Candiano, Bruschi et al. 2004). Gels were washed with water in order to remove the excess dye, and then scanned at 300 dpi in an Epson Expression 1680 Pro instrument (Adobe Photoshop program). The pI values of protein spots of interest were calculated according to the pH curve provided by the supplier of the immobilized pH gradient strips (<http://www.gelifesciences.com>). Molecular masses were calculated using the semilogarithmic curves of marker molecular mass (log) vs. migration distance corresponding to each of the gels analysed.

11.4. Protein digestion

Protein spots of interest were cut out from polyacrylamide gels, as well as control pieces from a protein-free region of the gel, placed in a 1.5 mL tube and washed 4 times with water. The gel pieces were washed with 0.5 mL of 100 mM EDTA for 15 minutes, with frequent vortexing. Supernatants were discarded and the gel pieces were dehydrated with 100% methanol for 5 min. The dehydrated gel pieces were rehydrated by incubation with 30% methanol, at RT. They were then washed twice with 1 mL ultra-pure water for 10 min, mixing frequently. The gel pieces were washed 6 times, for 20 min each time, with 50% methanol containing 20 mM diammonium phosphate (di-AP), until all traces of the Coomassie stain disappeared. After washing the pieces with water for 10 min, at RT, they were dried in a SpeedVac centrifuge for 30 min. Lastly, the dry gel pieces were rehydrated with a solution of 0.5 mg/mL trypsin in ammonium bicarbonate (pH 8.0) containing 10% acetonitrile and incubated overnight at 37°C. After trypsinization, supernatants were transferred to a clean tube and the remaining gel pieces were covered with 5% formic acid and incubated in a sonicating water bath for 20 minutes, in order to extract the peptides.

The supernatants therefrom were combined with the previous ones and dried in SpeedVac centrifuge, to be subjected to mass spectrometry analysis.

11.5. MALDI-TOF/TOF analysis and database searching

This work was a service by the Protein Network group of the ICGB, and is briefly described below. Trysin digests were resuspended with 50% acetonitrile-0.1% trifluoroacetic acid and spotted on stainless steel sample target plates. Peptide mass spectra were obtained on an Applied Biosystem Sciex 4800 MALDI TOF/TOF mass spectrometer. Data were acquired in positive MS reflector using a CalMix5 standard to calibrate the instrument (ABI4700 Calibration Mixture). Mass spectra were obtained from each sample spot by accumulation of 800 laser shots in an 800-4000 mass range. For MS/MS spectra, the 15 most abundant precursor ions per sample were selected for subsequent fragmentation by high-energy collision-induced dissociation. The collision energy was 1 keV and the collision gas was air. The criterion for precursor selection was a minimum S/N of 20. The interpretation of both the MS and MS/MS data was carried out using the GPS Explorer software (Applied Biosystems) that acts as an interface between the Oracle database containing raw spectra and a local copy of the MASCOT search engine. Peptide mass fingerprints obtained from MS analysis were used for protein identification in the Swiss-Prot or TrEMBL non-redundant databases. The data obtained were screened against human databases. All peptide mass values are considered monoisotopic and mass tolerance was set at 25-50 ppm. Trypsin was the digestion enzyme, one missed cleavage site was allowed, cysteine carboamidomethylated was assumed as a fixed modification, and methionine was assumed to be partially oxidized. MASCOT (Matrix Science) scores greater than 70 were considered significant ($p < 0.05$). For MS/MS analysis, all peaks with an S/N greater than 20 were searched against the SwissProt database considering the same modifications as in the MS database search, with a fragment ion mass tolerance of less than 0.5 Da.

12. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010. Differences between individual comparisons made in growth curves and protein expression data from Western blotting quantifications were analysed by one way ANOVA followed by Bonferroni's multiple comparison test. p -values < 0.05 were considered significant.

RESULTS

Chapter 1. Anti-mycobacterial activity and drug cytotoxicity

Anti-mycobacterial treatments are based on the combination of several drugs, which compared with mono-drug therapy have a lower probability of disease recrudescence and development of drug-resistant strains. Poor control of basic tuberculosis and inadequate treatments, associated with the absence of new drugs and strategies, led to the appearance of multi-drug resistant (MDR-TB) strains. These strains are resistant to Isoniazid and Rifampicin, both of which are established antibiotics for the treatment of tubercular disease. MDR-TB treatments are longer, costly, and induce more side effects than those against drug-susceptible strains (Di Perri and Bonora 2004).

The wrong management of non-susceptible strains led to the appearance of extensively drug resistance (XDR-TB). In addition to Isoniazid and Rifampicin, these strains are resistant to compounds of the quinolone family and to the second-line injectable drugs kanamycin, capreomycin and amikacin. This increases the time, cost and side effects of treatments. Up to date there are no guidelines available for the management of XDR patients requires the application of guidelines for MDR-TB (Jain and Mondal 2008; Madariaga, Lalloo et al. 2008). XDR-TB cases are commonly associated to HIV infection, which makes treatment difficult since synergism between both pathogens and drug interactions become additional issues to be carefully considered (Elston and Thaker 2008). Pharmaceutical companies have not shown sufficient interest in finding and/or developing new anti-tubercular compounds probably due to the low economic status of most of the affected countries. Rifampin, in use since 1966, is the last compound incorporated to anti-tubercular therapies. In conclusion, the appearance of resistant strains which are associated with extended treatments and the incompatibilities between anti-tubercular and anti-retroviral therapies call for the development of new anti-mycobacterial compounds.

Drug candidates for the fight against TB can arise from high throughput screenings or from chemical modification of compounds already in use, with the aim of maximizing their activity (Laurenzi, Ginsberg et al. 2007). Some interesting approaches are based on the design of compounds that can interfere with specific biochemical targets in the microorganisms. This has to take into consideration the mycobacterial metabolic status, since these microorganisms can enter a dormancy stage in which they evade the action of many antibiotics usually employed against infections (Sensi 1989; Murphy and Brown 2008). Another strategy, referred to as blind screening, is based on the isolation and identification of compounds with anti-mycobacterial activity from fermentation broths. In this way, several compounds such as streptomycin, kanamycin, capreomycin, and D-cycloserine have been isolated from different strains of *Streptomyces* *sps.* and successfully employed in anti-tubercular treatments (de Souza 2009). Natural product activities against tuberculosis have been extensively reported (Newton, Lau et al. 2000), confirming that they represent an excellent reservoir of novel compounds.

This thesis reports the study of the action of the synthetic compounds AC178, AC202, AC203, AC204, F2 and 9A, and the naphthoquinones of plant origin lapachol and β -lapachone against *Mycobacterium avium* (strain 485). Naphthoquinones in general and in particular lapachol, a substituted hydroxyl-naphthoquinone, show a wide spectrum of different biological activities (Guiraud, Steiman et al. 1994; Oliveira, Lemos et al. 2002; Sacau, Estevez-Braun et al. 2003; de Andrade-Neto, Goulart et al. 2004; Bezerra, De Oliveira et al. 2005; Breger, Fuchs et al. 2007; Lemos, Monte et al. 2007). The naphthoquinone plumbagin is the only one reported up to date to show activity against some mycobacteria in liquid culture (Mossa, El-Feraly et al. 2004). The other compounds the anti-mycobacterial activity of which was tested during the present work have all been produced by chemical synthesis.

1. Anti-mycobacterial activity (mycobacteria in liquid culture)

The anti-mycobacterial activities of the compounds tested on *M. avium* growing in liquid culture were determined by the microplate Alamar Blue assay (Franzblau, Witzig et al. 1998), which is based on the reducing ability of proliferating bacteria that results in an indicator colour change from blue to pink. The results obtained are summarized in Table 2. F2 was the most active compound with a MIC of 8 µg/mL, different from the value found by Banfi et al., who reported MICs ranging from 16 to 320 µg/mL against 17 different strains of *M. avium* (Banfi, Mamolo et al. 2001). The different sensibility to F2 here observed may be due to the use of a different strain of *M. avium*. The AC series compounds, lapachol and β-lapachone showed MICs ranging from 16 to 64 µg/mL. The extremely high MIC of the compound 9A (>512 µg/mL) reflected its lack of efficiency and excluded it from further studies.

MICs of the naphthoquinone lapachol against *S. aureus* and *S. typhimurium* were also determined, once it was established that this was a promising anti-mycobacterial agent, as described further below in this chapter 2 (sections 3 to 5). The value found for *S. aureus* (128 µg/mL / 528 µM) was the same or lower than that reported in the literature for the most susceptible strains (Oliveira, Miranda et al. 2001), and lower than that toward methicillin resistant *S. aureus* (Pereira, Machado Tde et al. 2006). Lapachol activity against *S. typhimurium* was studied here for the first time. The compound was not effective against *S. typhimurium*, with a MIC of 256 µg/mL (1 mM). The activities against these bacteria served as a control of the activity of the lapachol used during this work.

The MIC values obtained for the compounds tested, with the exception of 9A, were compatible with their potential use as anti-mycobacterial agents. Therefore, macrophage cytotoxicity studies were conducted.

Compound	Source or reference	<i>M. avium</i>	<i>S. aureus</i>	<i>S. typhimurium</i>
A9 ^a	Cateni, F, et al, (Cateni, Bonivento et al. 2007)	>512 (>1mM)	ND	ND
F2 ^a	Banfi, E. et al (Banfi, Mamolo et al. 2001)	8 (22µM)	ND	ND
AC178 ^b	Avidin Ltd.	64	ND	ND
AC201 ^b	Avidin Ltd.	32	128	ND
AC202 ^b	Avidin Ltd.	16	ND	ND
AC203 ^b	Avidin Ltd.	32	ND	ND
AC204 ^b	Avidin Ltd.	30	ND	ND
Lapachol ^c	Lapacho tree	32 (132µM)	128 (530µM)	>256 (>1mM)
β-lapachone ^c	Lapacho tree	16 (66µM)	ND	ND

Table 2. Susceptibility of *M. avium*, *S. aureus*, and *S. typhimurium* to putative antibiotics.

The lowest concentrations (µg/mL = mg/L) that arrested the growth of *M. avium*, *S. aureus* and *S. typhimurium* in liquid broth (Minimal Inhibitory Concentrations = MIC) were determined using the Microplate Alamar Blue Assay (MABA). MIC values ranged from 8 to 64. The compound A9 did not show any antimicrobial activity up to 512 µg/mL.

ND: Not determined

Bacteria used: *M. avium* strain 485, serovar 21, *S. aureus* strain 502A, *S. typhimurium* strain APGFP

a. A9 (1,2-*O*-diacyl-3-*O*-glucosylglycerol) and F2 (N1-(3-(4-CH₃)aryl-1-(pyridin-2-yl)-3-oxo-propyl)-2-pyridine-carboxamidrazone) were obtained from the Dept. of Biomedical Sciences, Microbiology Section, University of Trieste, Italy.

b. AC compounds series were obtained from Avidin Ltd. is a company based in Szeged, Hungary (www.avidinbiotech.com).

c. Lapachol and β-lapachone were obtained from the Dept. of Antibiotics, Federal University of Pernambuco (Brazil).

2. Cytotoxicity toward THP-1 macrophages: Cell morphology, apoptosis and necrosis

The usual length of therapies for sensitive *Mycobacterium tuberculosis* is at least 6 months, and this increases in the case of resistant strains (WHO 2009) or for treatments of non-tuberculous mycobacterial infections (Piersimoni and Scarparo 2008; Ballarino, Olivier et al. 2009; WHO 2009). Such long periods of treatment could result in relevant side effects. Some quinones, including lapachol and β -lapachone, are known to be cytotoxic to tumor cells (Boothman, Trask et al. 1989). If the effects produced by compounds under investigation involve the innate immune system, this could result in rather immediate consequences. In a first stage, it was investigated whether the compounds described in Table 2 are toxic to THP-1 macrophages at their MICs, macrophages being the cells used as hosts for *M. avium* during this work.

A preliminary evaluation of cytotoxicity was through the observation of changes in cell morphology. Changes in shape, adherence, cytoplasmic swelling or vacuolization/granulation constitute a reliable indicator of toxicity, especially in the case of macrophages, which are highly sensitive to environmental conditions. Morphology changes are shown in Figure 12, A, where it can be seen that the compounds β -lapachone, F2, AC178, AC202, AC203 and AC204, at their respective MIC significantly affected the cell size, shape and spreading. Absence of elongation, cell rounding, and shrinking were observed. Cells treated with F2 and AC202 were detaching. The compounds F2, AC178, AC202, AC203, AC204 and β -lapachone were therefore excluded from further experiments. Instead, untreated (control) and lapachol-treated cells showed similar characteristics. Size and granularity were not affected by lapachol (Fig. 12, B & C). Cells treated with AC201 were well elongated and adherent, but noticeably smaller (Fig. 12, B). In fact, the protein content per cell was found to be 0.39 and 0.19 mg/mL in control and AC201-treated cells respectively, in agreement with the microscopic observation results.

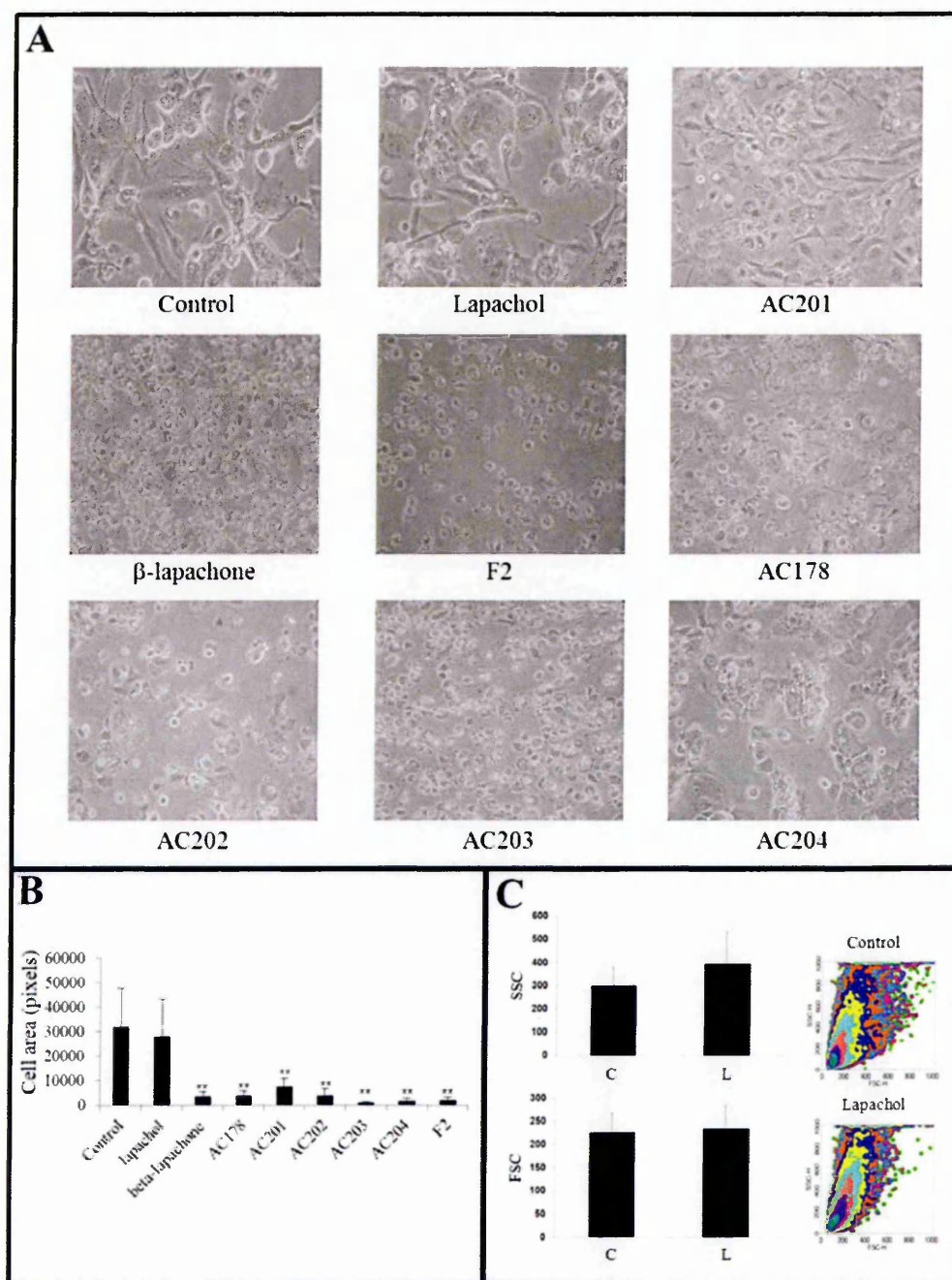


FIGURE 12. Effect of putative anti-mycobacterial compounds on THP-1 macrophage morphology.

A. Morphology of THP-1 macrophages either untreated (control) or treated for 4 days with lapachol, β -lapachone, F2, AC178, AC201, AC202, AC203 or AC204 at their MICs (listed in Table 2). Pictures correspond to inverted light microscopy images at 100 X magnification. With the exception of lapachol and AC201, the following changes were observed after treatment: absence of elongation, cell rounding, shrinking and detachment. Untreated and lapachol-treated cells presented an apparently similar morphology. AC201 induced cell size reduction.

B. Quantification of cell size by imaging. The size of 100 cells was determined, considering size as the area in pixels. Only cells treated with lapachol showed a normal size. All other compounds produced a significant reduction of cell size.

C. Quantification of cell size and granularity by FACS. Forward light scatter (FSC-H) and side light scatter (SSC-H) density plots of THP-1 macrophages and bar graphs of relative geometrical means of SSC and FSC. Results are expressed as means \pm SD (n=10). C: control; L: lapachol. Statistical analysis was by ANOVA (** $p \leq 0.01$, * $p \leq 0.05$)

Apoptosis studies were subsequently performed by flow cytometry analysis of annexin V binding. This analysis could only be performed regarding lapachol since the compound AC201 has an intrinsic fluorescence which interferes with the flow cytometry analysis. The result of three independent experiments showed that a similar number of annexin V-positive cells was present after treatment of THP-1 cells with lapachol (32 µg/mL) for four days, compared with untreated cells (Fig. 13, A). DNA fragmentation is a common event of apoptosis and necrosis, with temporal differences. It takes place in the early stages of apoptosis, before the loss of cell membrane integrity. Instead, in necrosis it is a late event that follows plasma membrane permeability changes (Fairbairn and O'Neill 1995). No DNA degradation and/or fragmentation was observed by agarose gel electrophoresis of the DNA isolated from untreated and lapachol- or AC201-treated cells (Fig. 13, B & C).

3. Intracellular anti-mycobacterial activity

Once the MICs of lapachol and AC201 toward *M. avium* growing in liquid culture and the absence of apoptotic or necrotic effects on the THP-1 macrophages that were to be used as host cells were established, it was essential to evaluate the activity of the two compounds against intracellular mycobacteria. This is a crucial point when the activity of a drug intended for intracellular microorganisms is tested, because permeability barriers, efflux pumps and/or metabolic events can diminish or even prevent the drug from reaching its target, reducing its capability to kill or arrest the growth of the intracellular organism, as shown by Tomioka et al (Tomioka, Sano et al. 2002).

THP-1 macrophage-like cells were infected with *M. avium* at a 30:1 ratio, allowing 4 hours to ensure maximum uptake of the microorganisms. Extra-cellular bacteria were washed off and the *M. avium*-containing host cells were cultured in the presence or absence of lapachol (16 or 32 µg/mL) or AC201 (32 µg/mL) for 4 or 7 days. At time 0, day 4 and day 7, host cells were lysed and plated on agar and the number of viable intracellular bacteria was determined by colony counting.

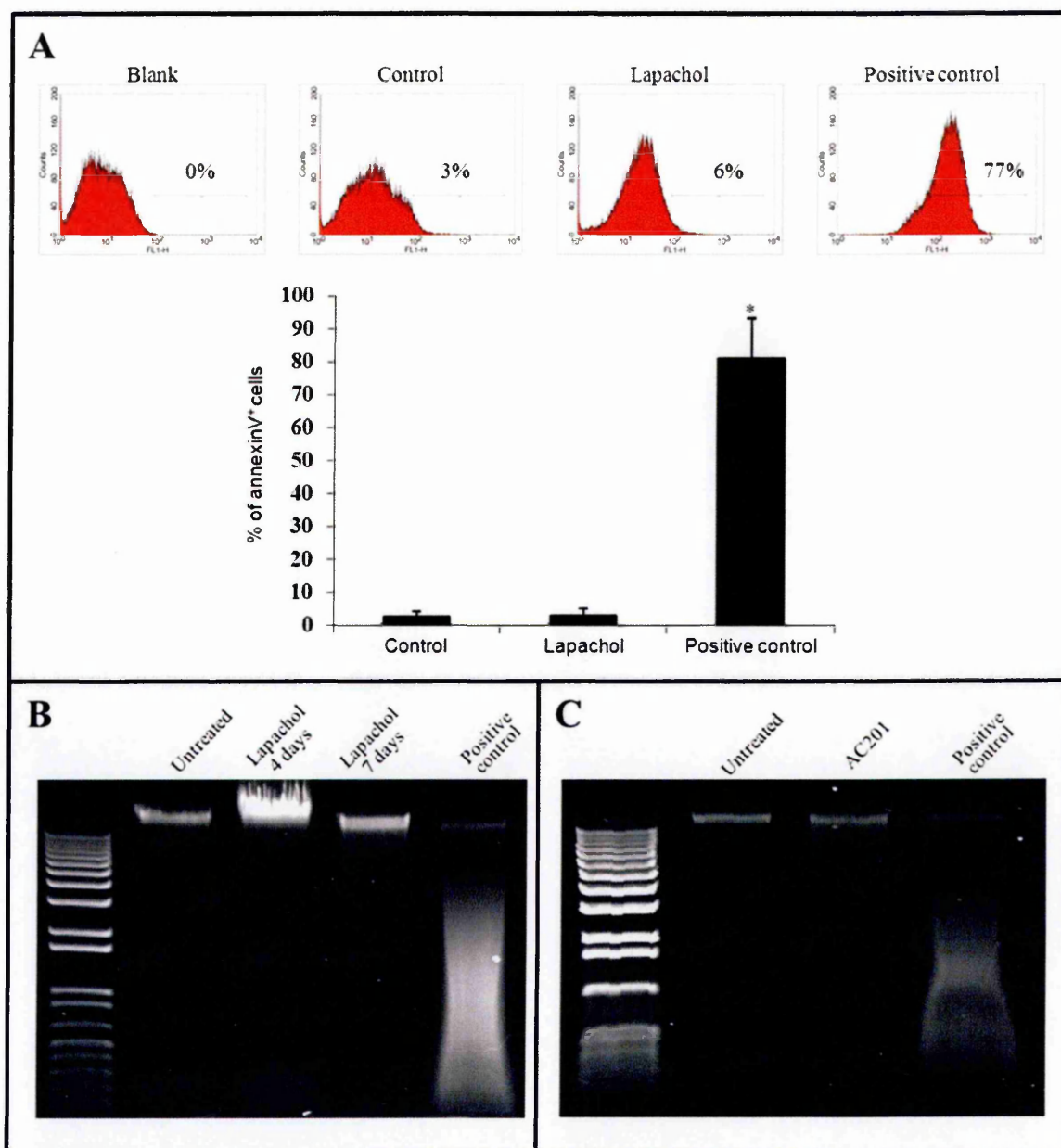


FIGURE 13. Lapachol and AC201 do not induce necrosis or apoptosis of THP-1 macrophages.

A. Annexin V binding. The binding of annexin V-FITC on the surface of cells cultured for 4 days in the absence or presence of lapachol (32 $\mu\text{g/mL}$) was determined by flow cytometry. The positive control was cells treated with etoposide (2 $\mu\text{g/mL}$) for 48 hours. The blank was cells not incubated with annexin V-FITC. Results are expressed as histograms and as bar graphs indicating the mean percentages of annexin V⁺ cells \pm S.E. (n=3, independent experiments). Statistical analysis was by ANOVA (* $p \leq 0,05$).

B. Influence of lapachol on DNA fragmentation. DNA was extracted from cells cultured for 4 or 7 days in the presence of lapachol (32 $\mu\text{g/mL}$) and run on 0.8% agarose gels (200–400 ng/lane). The positive control was cells treated with 8% DMSO for 24 hours. The absence of a smear in the lapachol lanes is an indication of DNA integrity.

C. Influence of AC201 on DNA fragmentation. DNA was extracted from cells after 4 days in the presence of AC201 (32 $\mu\text{g/mL}$) and run on 0.8% agarose gels (200 ng/lane). The positive control was cells treated with 8% DMSO for 24 hours. The absence of a smear in the AC201 lane is an indication of DNA integrity.

At day 7, the number of intracellular *M. avium* (CFUs) in the presence of lapachol at 32 or 16 µg/mL was 10 or 5 times smaller than in its absence, respectively. This decrease was only significant for 16µg/mL ($\frac{1}{2}$ MIC) (Fig. 14, A). The proliferation of intracellular *M. avium* was prevented by the naphthoquinone, but the number of intra-cellular bacteria at day 7, in the presence of lapachol, was similar to that at time zero (Fig. 14, A). This is an indication of bacteriostasis rather than of bacterial killing.

In the presence of AC201 at 32 µg/mL, growth of *M. avium* was decelerated (Fig. 14, B). However, at day 7 bacterial numbers were still 10-fold higher than at time 0, reflecting the limited efficiency of this compound in an intra-cellular environment.

As a reference, the intra-cellular proliferation of *M. avium* was determined under the effect of the first line anti-mycobacterial compounds clarithromycin, rifampicine and ethambutol at the concentrations that have been reported as average serum concentrations 24 h after their administration, namely 0.56, 1.60 and 0.35 µg/mL, respectively (Tomioka, Sano et al. 2002). At those low concentrations, these classical antibiotics just retard the growth of *M. avium*, higher concentrations being necessary for them to exert full activity (Fig. 14, C).

4. Distinguishing between bactericidal or bacteriostatic activity of lapachol

As described in the preceding section, lapachol was able to arrest the intracellular growth of *M. avium* at 32 µg/mL (132 µM), and to a lesser extent at 16 µg/mL (66 µM). It is known that a compound can be either bacteriostatic or bactericidal depending on the concentration used, according to the microorganism susceptibility (Rahal and Simberkoff 1979). Since the interaction between lapachol and the cell environment could interfere with its anti-mycobacterial activity, time courses of *M. avium* growth were performed in Middlebrook 7H9 broth, in the absence or presence of the drug at 32µg/mL. Aliquots taken during this time course were diluted and plated on agar for CFUs counting.

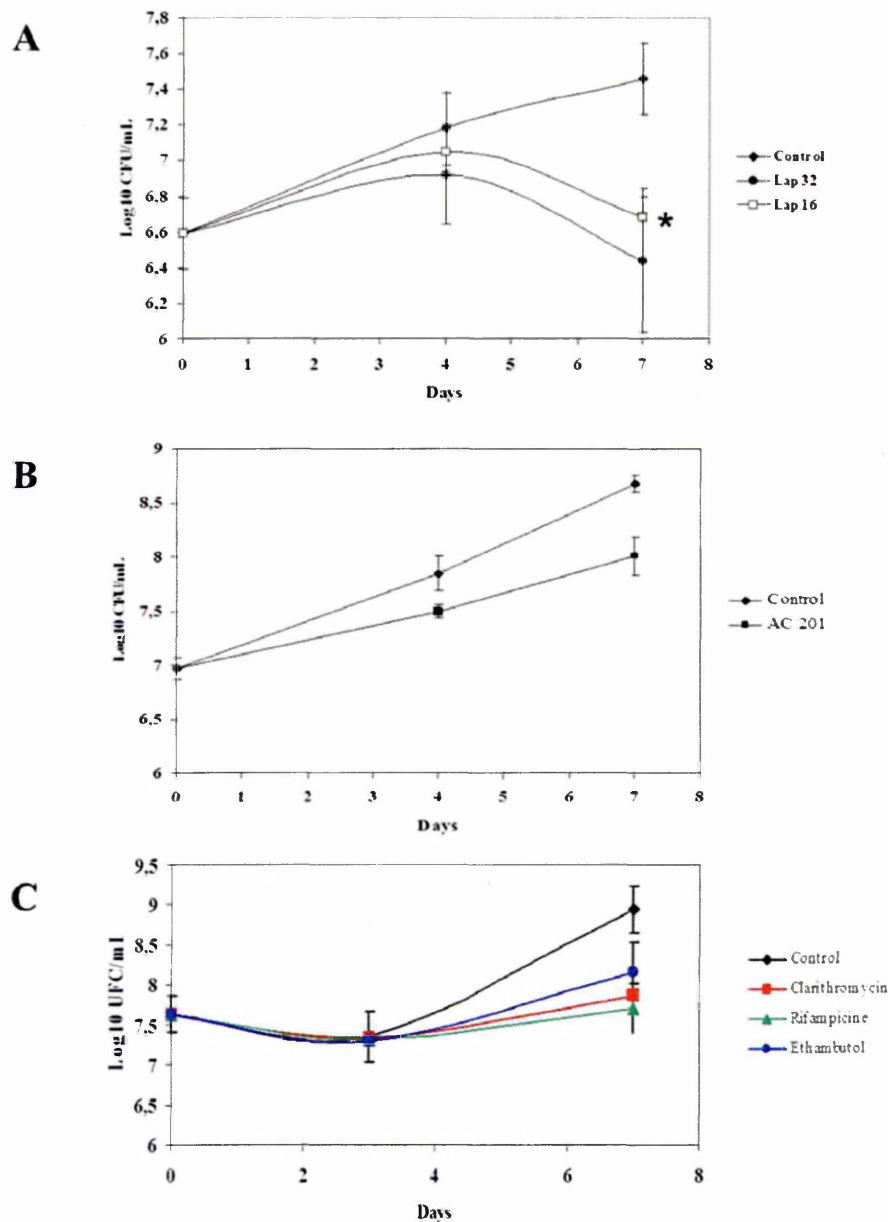


FIGURE 14. Anti-*M. avium* activity of lapachol (A) , AC201 (B), and three first line antibiotics (C) against intracellular *M. avium*, within THP-1 macrophage-like cells.

Growth curves of *M. avium* (strain 485, serovar 21) within THP-1 macrophages treated or not with 16 or 32 $\mu\text{g/mL}$ lapachol (A); 32 $\mu\text{g/mL}$ AC201 (B); 0.56 $\mu\text{g/mL}$ clarithromycin, 1.6 $\mu\text{g/mL}$ rifampicine or 0.35 $\mu\text{g/mL}$ ethambutol (C). Results are expressed as $\log_{10}\text{CFU/mL}$ (mean \pm S.E., $n=3$, independent experiments), normalized according to the protein content of each sample, which is equivalent to cell number. * Statistically significant (ANOVA, $p \leq 0.05$) reduction of intracellular *M. avium* CFUs compared with those in macrophages not treated with lapachol.

The results shown in Fig. 15 indicate a bacteriostatic effect of lapachol, with an invariant, low number of viable colonies until day 3 and no evidence of bacterial killing. The fact that growth of the organisms was observed from day 3 to 7 is probably due to lapachol inactivation or metabolism in the bacterial broth.

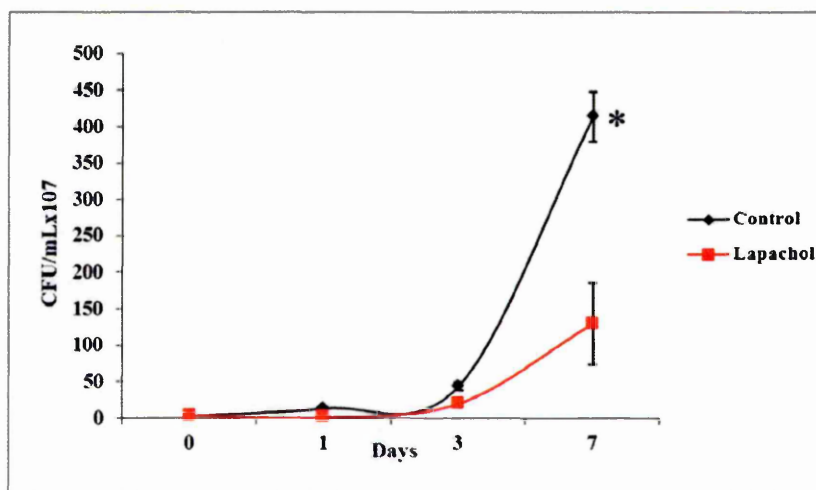


FIGURE 15. Bacteriostatic effect of lapachol toward *M. avium*

M. avium was grown until exponential phase in Middlebrook 7H9 broth at 37°C, with constant agitation and with or without 32 µg/mL (132 µM) lapachol added at time zero. Aliquots were taken at time 0, day 1, 3 and 7, diluted and plated on agar for CFU counting. Bacterial replication was very slow until day 3. From day 3 to 7 bacterial growth in the presence of lapachol was considerably slower than that of the control culture. The proliferation observed from day 3 to 7 in the presence of lapachol could be ascribed to a loss of drug activity in the broth. There is no evidence of a bactericidal effect of lapachol against *M. avium* at 132 µM.

The graph represents the result of three different independent experiments (n=3). Statistical analysis was performed by ANOVA (* $p \leq 0.05$ corresponds to the comparison of CFUs in the absence or presence of lapachol, at day 7).

The bacteriostatic or bactericidal character of the compound AC201 was not examined because this drug showed a scarce intracellular activity (see above: section 3; Fig. 14, B) and therefore it did not appear to be a promising drug candidate, as studies described further below demonstrate (Chapter 2, section 1).

Low MICs mean a better probability of *in vivo* applications, since side effects would be minimized. However, safe doses are related to the toxicity of the drug. For drugs to reach the site of action, they have to go through several biological barriers. They must be absorbed, distributed via the circulation system and finally arrive at tissues. Regarding mycobacterial infection, an active compound must also be able to enter the host cells and cross the phagosome membrane or reach somehow the phagosome where the microorganism resides. During this process, the putative anti-mycobacterial agent will be subjected to the cellular mechanisms directed at the elimination of foreign substances (Van Bambeke, Balzi et al. 2000; Van Bambeke, Michot et al. 2003; Goossens 2009). For that reason, MIC values will usually translate into higher *in vivo* doses. High doses imply an increase in the probability of side effects and toxicity. Still, a compound with a low MIC can be a poor *in vivo* performer, or one with a high MIC may be very efficient in the cellular environment and the dose necessary *in vivo* can even be lower than its MIC.

The MIC of the compounds tested here regarding *M. avium* growth in liquid broth ranged from 8 to 64 µg/mL (Table 2). These values are relatively low or comparable with MICs induced by a number of other natural compounds against mycobacterium sp. (Jimenez-Arellanes, Meckes et al. 2003; Borges-Argaez, Canche-Chay et al. 2007; Gordien, Gray et al. 2009; Kuete, Tangmouo et al. 2009; Molina-Salinas, Borquez et al. 2010), and higher than the MICs reported for plumbagin, a naphthoquinone analog to lapachol, totarol, ferruginol, ferulenol, and ferulenol acetate in relationship with some mycobacterium sp. (Mossa, El-Feraly et al. 2004; Kuete, Tangmouo et al. 2009).

As to the mechanism of action, lapachol probably interferes with bacterial cytochrome bc systems, a known property of naphthoquinones and in particular those with branched alkyl side-chains like lapachol (Kumagai 1997; Kessl, Moskalev et al. 2007). It could also induce bacterial oxidative stress, which is associated to the redox-cycling properties of

naphthoquinones, as reported for plumbagin and menadione (Guerra-Lopez, Daniels et al. 2007).

The cytotoxicity of lapachol toward different tumour cell lines has been studied (Rao, McBride et al. 1968; Oliveira, Lemos et al. 2002; Eyong, Kumar et al. 2008), since this compound was originally intended for the treatment of soft tissue tumours. Leukemic cells lines seem to be more sensitive to lapachol than solid tumors and low concentrations, ranging from 16 to 25 μM , have been reported to be cytotoxic (Salustiano, Netto et al. 2009). Our experiments showed no toxicity when THP-1 macrophage-like cells were treated with lapachol at 32 $\mu\text{g/mL}$ (132 μM), in agreement with Teixeira et al (Teixeira, de Almeida et al. 2001). These authors investigated the anti-leishmanial activity of lapachol and determined that this naphthoquinone only induced cytotoxicity of mouse peritoneal macrophages when its concentration was above 0.1 mg/mL (413 μM).

The fact that neither lapachol nor AC201 were toxic toward THP-1 macrophages at their anti-*M. avium* MIC meant that studies of their activity against intracellular organisms could be conducted, the results of which are described in Section 3 above.

The antimicrobial effect of lapachol against intracellular *M. avium* was here found to be more efficient than that toward bacteria growing in liquid broth. The intracellular proliferation of *M. avium* was arrested by lapachol at 16 $\mu\text{g/mL}$ (66 μM), i.e. half of its MIC. It can be speculated that the better intracellular activity of lapachol could be due to its lipophilic nature (Lira, Sester et al. 2008); to its being somehow concentrated within macrophages if it is not easily cleared from cytoplasm by efflux pumps or metabolizing systems; to its intracellular conversion into a more active derivative; to a higher stability within the phagolysosomal environment that provides protection against cytoplasmic clearing processes; to its superoxide generation activity (Bachur, Gordon et al. 1979) that could in turn react with NO and result in the generation of mycobactericidal peroxynitrate (Bogdan, Rollinghoff et al. 2000); or to a hypothetical inhibition of bacterial NO

reductase/s (NORs) that normally function as detoxifiers of macrophage-produced NO (Watmough, Field et al. 2009) The latter is supported by the fact that some bacterial NORs consist of cytochrome *bc* complexes (Suharti, Heering et al. 2004), and lapachol is able to interact with such complexes (Kessl, Moskalev et al. 2007). Another explanation for the higher intracellular activity of lapachol is linked to the fact that, as will be described in Chapter 3, lapachol affects some macrophage responses in a way that improves the capacity of the host cell to control the intracellular proliferation of mycobacteria. Therefore, there would be a combination of the intrinsic anti-mycobacterial activity of lapachol with a modulating effect on host cell responses.

As opposed to lapachol, AC201 was not satisfactory at arresting the intracellular growth of *M. avium*, although both drugs arrested *M. avium* growth in liquid broth with the same MIC. This illustrates how drug performance can differ depending on environmental conditions, in the present case those provided by the host cell.

The lack of induction of cell death by lapachol and AC201 was the starting point for the investigation of their influence on macrophage functions in relationship with some aspects of cellular immunity.

Chapter 2. Macrophage functions in response to activation

The considerable length of the treatments needed for mycobacterial infections (> 6 months), primarily intended to eliminate or control the growth of microorganisms, could have consequences at host cell and immune system level. Macrophage functions could be up- or down-regulated by the anti-mycobacterial agents being used, and this could have detrimental or beneficial effects. Therefore, knowledge on if and which macrophage functions are modulated is important to predict and consequently assess possible side effects in successive *in vivo* investigations. Macrophages are fundamental within the innate immune system since they generate reactive oxygen and nitrogen species, secrete mediators and cytokines, are able to phagocytose and degrade, and can present antigens. Basically, these cells provide a link between innate and adaptive immunity. Impairments of some of its functions could compromise the elimination of pathogens other than mycobacteria that the body may encounter during the long course of treatment to fight mycobacteria. Moreover, therapeutical anti-mycobacterial compounds could disturb cellular homeostasis, for instance inducing endoplasmic reticulum (ER) or oxidative stress. ER controls protein folding and the repair or elimination of missfolded proteins, and severe ER stress can trigger apoptosis (Tao, Ruan et al. 2009). The generation of oxidative and nitrosative metabolites is used by the cells to kill pathogens. However, host cell molecules such as lipids, proteins and DNA can be also oxidized, and apoptosis could be triggered (Roberts, Laskin et al. 2009).

In order to determine whether the anti-mycobacterial compounds being evaluated here, namely AC201 and lapachol, have any influence on macrophage responses to stimuli, we used resting or IFN- γ -activated cells, cells agonised with the TLR2 lipopeptide P₃CSK₄ and in some cases *M. avium*-infected macrophages. IFN- γ is the main macrophage-activating cytokine (Schroder, Hertzog et al. 2004) and TLR2 is a major innate immune

response mediator recognizing the broadest range of bacterial molecules (Takeuchi, Sato et al. 2002; Zahringer, Lindner et al. 2008).

All the experiments described and commented in sections 1 to 7 of this chapter were performed with THP-1 cells that became macrophage-like after 4 days of differentiation under the effect of 20 nM phorbol-myristate acetate (PMA). These macrophage-like cells were left untreated (controls) or were treated with 300 nM P₃CSK₄ or 100 U/mL IFN- γ for 4 days, i.e. until day 8. In some cases, the macrophages were infected with *M. avium* (4 hr internalization) and then kept in culture for 4 days. At day 8, lapachol (32 μ g/mL), compound AC 201 (32 μ g/mL) or first line TB antibiotics (clarithromycin, rifampicin or ethambutol) were added or not, and cells were cultured for 4 additional days, i.e. until day 12. In brief, activating or infecting agents were present from day 4 till day 12 in all cases. Lapachol, AC201 or first line antibiotics were present from day 8 till day 12. A graphic scheme of this working protocol is shown in Figure 16.

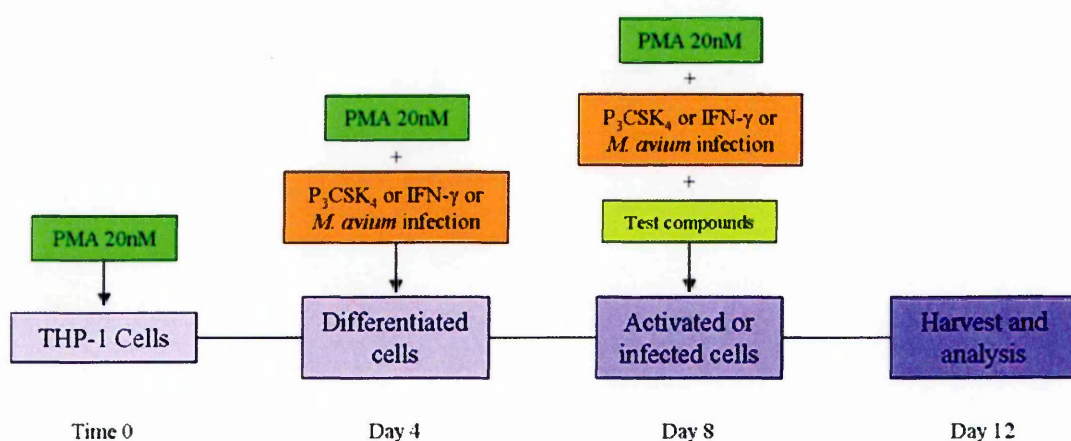


FIGURE 16. Experimental scheme of THP-1 differentiation and subsequent treatments of THP-1 macrophages.

THP-1 macrophages were incubated in 6-well plates with 20nM PMA for 4 days, at 37°C. At this point cells were activated by addition of P₃CSK₄ or IFN- γ , or infected by *M. avium*, and incubated for 4 additional days to allow the establishment of intra-cellular infection. At day 8 cells were treated or not with test compounds for 4 additional days, after which they were harvested for analysis.

After pathogens invade tissues, they can bind to a wide range of pattern recognition receptors (PRRs) abundant on phagocytic cells. Some of the PRRs actually mediate phagocytosis, i.e. the means through which pathogens can gain access to the intracellular environment. Pathogen surface components (PAMPs, pathogen-associated molecular patterns) are recognized by a variety of phagocytic receptors on the effector cell such as complement receptor 3, CD14, Fc, mannose and scavenger receptors, etc, promoting microorganism uptake. For some pathogens, mycobacteria among them, the route of entry determines their fate in the intracellular environment. For instance, oxidative stress could or not be triggered (Ernst 1998). Internalized pathogens are confined in cytosolic vesicles called phagosomes, which acquire degradative enzymes (hydrolases, proteases) after fusing with lysosomes, giving rise to phagolysosomes. Proton-ATPase pumps present in cytoplasmic vesicles also fuse with phagosomes and acidify their luminal space, generating the right conditions for some enzymes to be active (Sun-Wada, Wada et al. 2003).

TLRs are transmembrane receptors, and PAMP binding triggers signaling cascades leading to the secretion of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IL-12 (Kaisho and Akira 2006; Kumar, Kawai et al. 2009). TLRs do not mediate phagocytosis but the establishment of adaptive immunity, and can induce inflammation. The cytokines secreted act on surrounding cells and induce the secretion of chemotactic cytokines or chemokines such as monocyte chemoattractant protein-1 (MCP-1 or CCL2), that is known to attract monocytes to the site of inflammation (Deshmane, Kremlev et al. 2009). In order to migrate, inflammatory cells adhere to endothelial cells via molecules such CD54 or CD87. Local proteolysis by matrix metalloproteinases facilitates diapedesis through vessel walls (van de Stolpe and van der Saag 1996; Plesner, Behrendt et al. 1997). The monocytes recruited to the inflamed tissue differentiate into macrophages that can become activated, recognize pathogen via PRRs and deal with the organisms. Internalized pathogens can be killed due to the generation of reactive oxygen species (ROS) and nitrogen reactive

intermediates (NRI). An oxidative phase takes place initially, after the switching-on of NADPH oxidase, followed by a prolonged nitrosative phase during which iNOS activity results in the inhibition of bacterial growth (Raupach and Kaufmann 2001; Bhatt and Salgame 2007).

Activation of macrophages by IFN- γ is essential to fight against intracellular pathogens. IFN- γ is the main macrophage activating cytokine and boosts the cellular machinery to increase its capacity to generate ROS and NRI and to express MHC molecules, among other effects. This enhances bacterial killing and antigen presentation by macrophages (Korbel, Schneider et al. 2008; Billiau and Matthys 2009) in combination with stimulating agents, e.g. LPS (Fang 2004).

Pathogenic mycobacteria have developed strategies to subvert the cellular machinery that controls pathogen growth, and can therefore persist in the host cells. They efficiently prevent phagosome maturation (Via, Deretic et al. 1997; Philips 2008), which results in phagosomes that are less acidified and in an impaired antigen presentation (Noss, Pai et al. 2001; Hestvik, Hmama et al. 2005). Mycobacterium-infected macrophages also secrete of anti-inflammatory cytokines (IL-10, TGF- β), which suppress immune responses and facilitate the intracellular survival and replication of the microorganisms within phagosomes, with the result of promoting chronic infection (Bogdan, Vodovotz et al. 1991; Ferrara, Bleck et al. 2008).

Physiologically generated ROS and NRI also play an important role as second messengers in different tissues (Kamata and Hirata 1999; Rhee 1999; Wang-Rosenke, Neumayer et al. 2008). Although an increased generation of these radicals is directly associated with improved pathogen killing, ROS and NRI are highly reactive and can damage biomolecules if they overcome the cellular antioxidant system, and can eventually induce cell death. In order to protect themselves against the deleterious effects of oxidative stress, macrophages and bacteria respond employing antioxidant systems based on the

activity of superoxide dismutases, peroxidases, catalases and degradative enzymes that scavenge the reactive species (Bogdan, Rollinghoff et al. 2000; Ferret, Soum et al. 2002).

Natural products have been described to be anti-oxidant or pro-oxidant agents, protecting or harming cells. For instance, polyphenols such as quercetin and resveratrol have been described to control excessive oxidative stress (de la Lastra and Villegas 2007; Kumar, Sehgal et al. 2008). Quinones, e.g. plumbagin and lapachol, are able to generate reactive oxygen species by reducing oxygen to superoxide (Hernandez-Munoz, Gomez et al. 2009). This is considered to be an important mechanism to eliminate cancer cells (Srinivas, Gopinath et al. 2004).

Here are reported the effects of lapachol on the surface expression of TLR2, CD14 and phagocytic receptors; in the levels of oxidative, nitrosative and ER stress proteins and in the secretion of some cytokines in/by THP-1 macrophages either resting or after TLR2 agonism by the lipopeptide P₃CSK₄, IFN- γ treatment or *M. avium* infection. The effect of the compound AC201 was only examined regarding ER and oxidative stress, since a cytoskeleton-altering effect was found at an early stage of the studies that led to the interruption of investigations on AC201 (see last paragraph of this section).

The analysis of the expression of the surface receptors TLR2, CD14, CD18, ManR, Fc receptors (CD16, CD32, and CD64) and scavenger receptors was performed by flow cytometry. The expression of proteins involved in oxidative, nitrosative and ER stress was quantified by immuno-blotting, and cytokine secretion was determined by ELISA. Protein concentrations were accurately measured, on the basis of which equal amounts of protein from the different samples being compared were loaded on gels to be immuno-blotted. Actin and/or GAPDH were quantified on the same blotting membrane as the proteins being quantified, to detect possible differences in loading and/or blotting.

Measurement of actin proved misleading since levels of cytosolic, but not membrane-associated, actin were found to be highly variable, particularly under the influence of the

compound AC201 (Fig. 17, C). This fact led to the realization that AC201 was altering actin dynamics, which was the reason to discontinue its study, together with its poor control of intracellular *M. avium* growth (see Chapter 1, section 3). GAPDH was successively used as a sample loading/blotting indicator (sections 2 to 4). The expression variability of this marker was found to be 7-15%.

1. Endoplasmic reticulum (ER) stress

ER is the cellular compartment responsible for protein folding. Glucose deprivation, viral infection and an aberrant Ca^{2+} regulation can induce the accumulation of unfolded proteins, triggering an ER unfolded protein response (UPR) aimed at restoring ER normal function. UPR are orchestrated by chaperones such as Grp78. This multifunctional protein is a central regulator of ER homeostasis (Xu, Bailly-Maitre et al. 2005; Li, Ni et al. 2008), and its up-regulation is considered an ER stress marker. P_3CSK_4 treatment (TLR2 agonism) induced a significant decrease in Grp78 (Fig. 17, A), while neither AC201 nor lapachol induced ER stress (Figs. 17, A and C). AC201 actually produced a significant decrease in Grp78 levels in cells treated with both the TLR2 agonist and IFN- γ . The effect of *M. avium* infection on ER stress is shown in Fig. 17, B. Infection did not induce any increase in Grp78 levels. When infected cells were incubated with lapachol, the levels of Grp78 were significantly decreased ($p \leq 0.05$).

2. Oxygen metabolism

Oxygen metabolites are produced by macrophages and other phagocytes to kill pathogens in general (Bogdan, Rollinghoff et al. 2000), although their role in mycobacterial killing is not clear

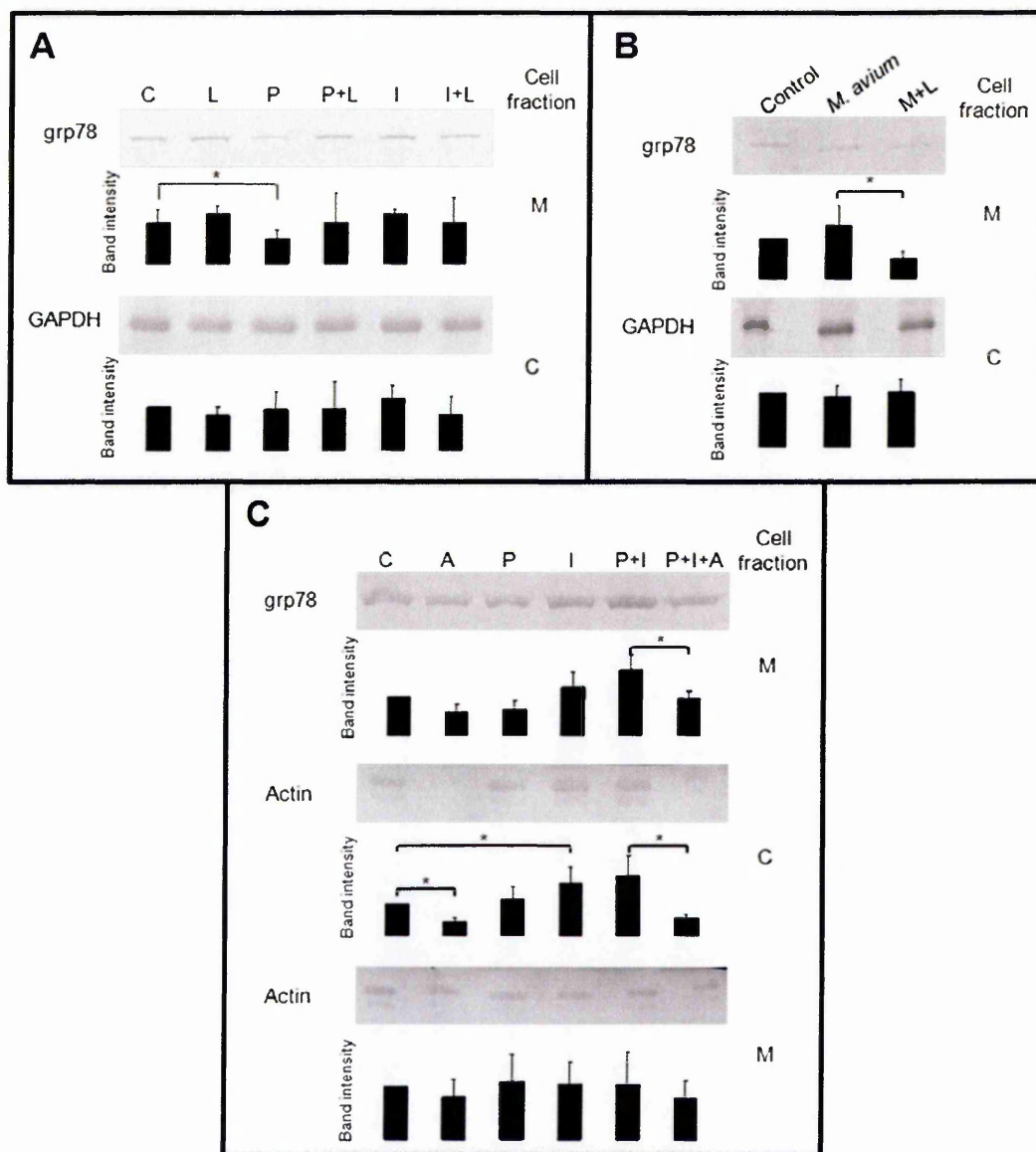


FIGURE 17. Effect of activation, *M. avium* infection and treatment with lapachol or AC201 on the endoplasmic reticulum (ER) stress of THP-1 macrophages.

Macrophages were treated with the stimuli indicated above for 4 days, then cultured for 4 additional days in the absence or presence of lapachol or AC201. Cells were fractionated in membranes (M) and cytosol (C). Membrane and cytosolic fractions were electrophoresed and western blotted onto nitrocellulose, and the blotting membranes probed with antibodies against the ER stress marker grp78 (membrane), GAPDH (cytosol) or actin (cytosol and membrane). Band intensities were determined using ImageJ software.

A. Macrophages treated with P₃CSK₄ or IFN- γ , in association or not with lapachol (32 μ g/mL)

C: Control; L: lapachol; P: P₃CSK₄; P+L: P₃CSK₄ + lapachol; I: IFN- γ ; I+L: IFN- γ + lapachol

B. Macrophages infected with *M. Avium*, then treated or not with lapachol (32 μ g/mL)

M+L : *M. avium* + lapachol

C. Macrophages treated with P₃CSK₄ or IFN- γ , then treated or not with AC201 (32 μ g/mL)

C: Control; A: AC201; P: P₃CSK₄; I: IFN- γ ; P+I: P₃CSK₄+IFN- γ ; P+I+A: P₃CSK₄+IFN- γ +AC201

Results are expressed as means \pm S.E. (n=3, independent experiments). Significant differences ($p \leq 0.05$) in the comparisons indicated by brackets are shown by the symbol *.

GAPDH and actin were intended as controls of electrophoresis loading and western blotting. Actin turned out to be too variable and its use as loading marker was therefore discontinued.

due to the fact that these bacteria express superoxide dismutase (Raupach and Kaufmann 2001). The generation of O_2^- is catalyzed by the NADPH oxidase complex after its activation by microbial products, e.g. LPS. When activation is primed by IFN- γ , it can reach higher levels. O_2^- can subsequently be converted in H_2O_2 by superoxide dismutase or to hydroxyl radical ($\bullet OH$) through the Fe^{+3} -catalyzed Haber-Weiss reaction (Bogdan, Rollinghoff et al. 2000). In the presence of NO generated by iNOS, O_2^- can generate peroxynitrite (Bartosz 1996; Guzik, Korbuet et al. 2003).

During the present work, the capacity of NADPH oxidase to generate O_2^- was evaluated by determining by immunoblotting the levels of two inducible proteins: catalytic, membrane-bound gp91^{phox} and cytosolic, regulatory p47^{phox}, which docks to gp91^{phox} after TLR agonism (El-Benna, Dang et al. 2008; El-Benna, Dang et al. 2009). Superoxide generation was not determined directly in view of the strong possibility of interference due to the ability of quinones, among them lapachol, to generate oxygen metabolites (Docampo, De Souza et al. 1978; Bolton, Trush et al. 2000; Goulart, Falkowski et al. 2003). The superoxide produced could in turn consume NO, as explained above.

As expected, a significant increase in the expression of gp91^{phox} and p47^{phox} was observed when THP-1 macrophages were TLR2-agonised (Figs. 18, A & C). In the case of IFN- γ treated cells, only an increase in the levels of p47^{phox} was observed (Fig. 18, C).

AC201 significantly reduced the levels of p47^{phox} in TLR2/IFN- γ -activated cells, and those of gp91^{phox} in both resting and TLR2/IFN- γ -activated cells (Fig. 18, C), suggesting a possible negative influence of this compound on the superoxide generation capacity of macrophages.

Lapachol significantly enhanced the induction of p47^{phox} by TLR2 agonism (Fig. 18, A). In association with *M. avium* infection, lapachol significantly reduced the levels of gp91^{phox} and p47^{phox} in the membrane fraction to resting cell values (Fig. 18, B), whereas the significant up-regulation of cytosolic p47^{phox} elicited by *M. avium* was not affected by the naphthoquinone.

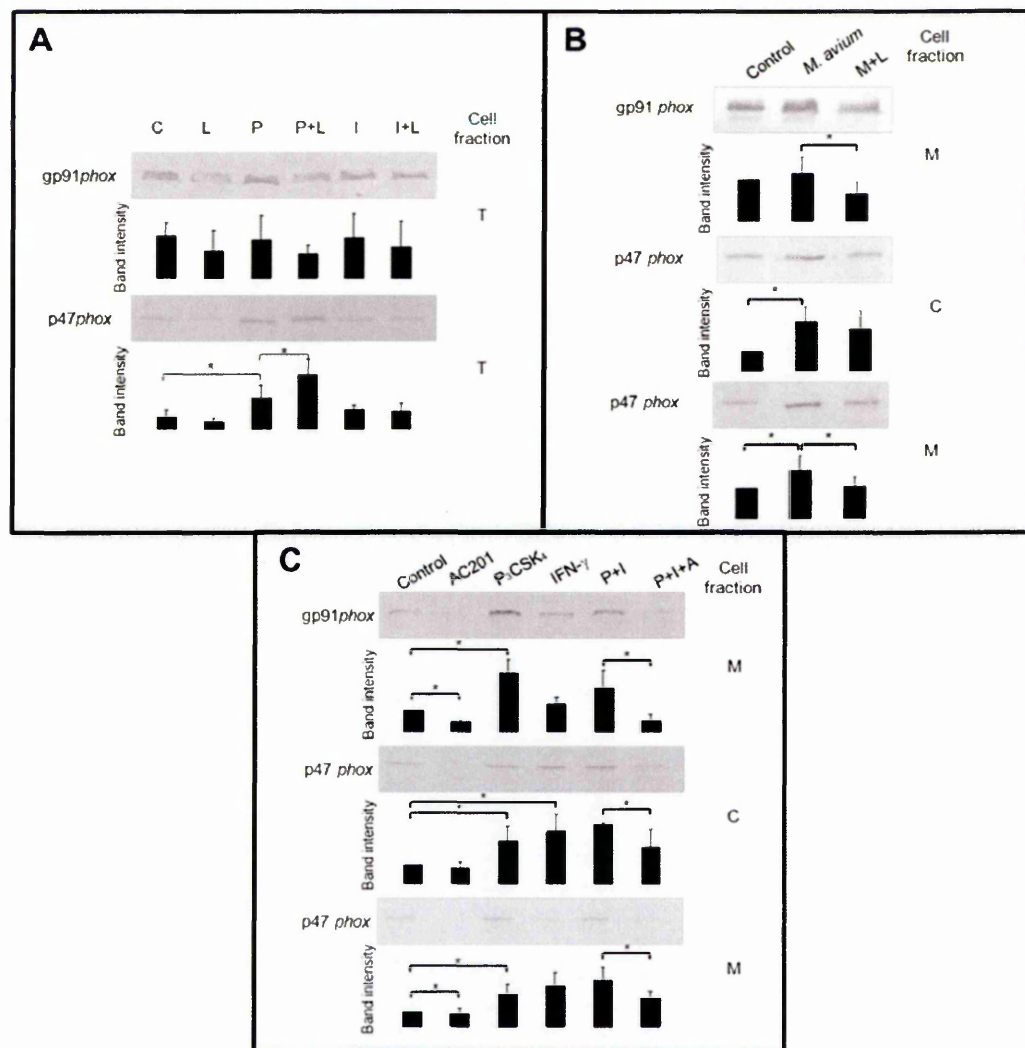


FIGURE 18. Effect of activation, *M. avium* infection and treatment with lapachol or AC201 on the expression of key proteins of oxygen metabolism by THP-1 macrophages.

Macrophages were treated with the stimuli indicated for 4 days, then cultured for 4 additional days in the absence or presence of lapachol or AC201. In **A**, total cell extracts (T) were used. In **B** and **C**, cells were fractionated in membranes (M) and cytosol (C). Total cell extracts, cytosolic or membrane proteins were electrophoresed, western blotted onto nitrocellulose and the blotting membranes probed with antibodies against the inducible components of NADPH oxidase gp91^{phox} and p47^{phox}. Band intensities were determined using imageJ software.

A. Macrophages treated or not with P₃CSK₄ and IFN- γ , in association or not with lapachol (32 μ g/mL)

C: Control; L: lapachol; P: P₃CSK₄; P+L: P₃CSK₄+lapachol; I: IFN- γ ; I+L: IFN- γ +lapachol

B. Macrophages infected with *M. avium*, then treated or not with lapachol (32 μ g/mL)

M+L: *M. avium* + lapachol

C. Macrophages treated or not with P₃CSK₄ or IFN- γ , then incubated or not with AC201 (32 μ g/mL).

C: Control; A: AC201; P: P₃CSK₄; I: IFN- γ ; P+I: P₃CSK₄+IFN- γ ; P+I+A: P₃CSK₄+IFN- γ +AC201

Results are expressed as means \pm S.E. (n=3, independent experiments). Significant differences ($p \leq 0.05$) in the comparisons indicated by brackets are shown by the symbol *.

3. Oxidative stress

Damage to the cell host caused by reactive oxygen species is controlled by scavenging enzymes, namely the mitochondrial MnSOD and the cytosolic CuZnSOD (Zelko, Mariani et al. 2002).

The TLR2 agonist P₃CSK₄ significantly increased the levels of MnSOD and this effect was not affected by either lapachol or AC201 (Figs. 19, A & C). CuZnSOD is constitutively expressed and its expression was not affected by any of the treatments (Fig. 19, A).

M. avium infection induced a significant up-regulation of MnSOD, which was not affected by the association of infection and lapachol (Fig. 19, B). Again, levels of the constitutively expressed CuZnSOD were not affected by infection or by lapachol treatment.

4. L-arginine metabolism

The level of expression of iNOS (NOS2), the enzyme generating NO from L-arginine, was significantly up-regulated by P₃CSK₄ (Fig. 20, A). Levels of iNOS in resting and TLR2 treated cells were not affected by lapachol.

The expression of arginase-1, a negative regulator of iNOS, was significantly down-regulated by TLR2 agonism, IFN- γ activation (Fig. 20, A) and *M. avium* infection (Fig. 20, B), and these changes were not influenced by lapachol. Downregulation of the expression of arginase-1 results in driving the use of arginine by iNOS, facilitating the production of NO by activated macrophages (Durante, Johnson et al. 2007).

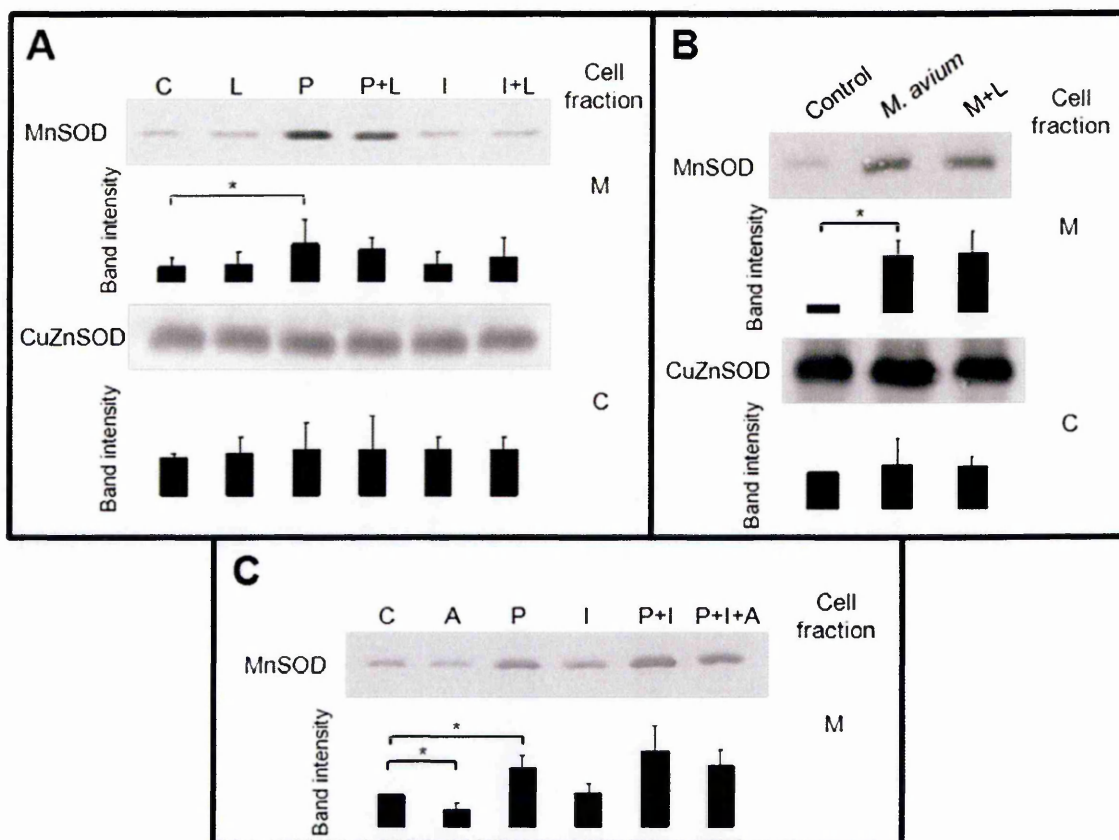


FIGURE 19. Effect of activation, *M. avium* infection and treatment with lapachol or AC201 on MnSOD and CuZnSOD expression by THP-1 macrophages.

Macrophages were treated with the stimuli indicated for 4 days, then cultured for 4 additional days in presence of lapachol or AC201. Membrane (M) and cytosol (C) fractions were electrophoresed, transferred to nitrocellulose membrane and western blotted, and the blotting membranes probed with antibodies against mitochondrial superoxide dismutase (MnSOD) (M) or CuZnSOD (C). Band intensities were determined using imageJ software.

A. Macrophages treated with P_3CSK_4 or IFN- γ , in association or not with lapachol (32 μ g/mL)

C: Control; L: lapachol; P: P_3CSK_4 ; P+L: P_3CSK_4 + lapachol; I: IFN- γ ; I+L: IFN- γ + lapachol

B. Macrophages infected with *M. avium*, then treated or not with lapachol (32 μ g/mL)

M+L: *M. avium* + lapachol

C. Macrophages treated with P_3CSK_4 or/and IFN- γ , in association or not with AC201 (32 μ g/mL)

C: Control; A: AC201; P: P_3CSK_4 ; I: IFN- γ ; P+I: P_3CSK_4 + IFN- γ ; P+I+A: P_3CSK_4 + IFN- γ + AC201

Results are expressed as means \pm S.E. (n=3, independent experiments). Significant differences ($p \leq 0.05$) in the comparisons indicated by brackets are shown by the symbol *.

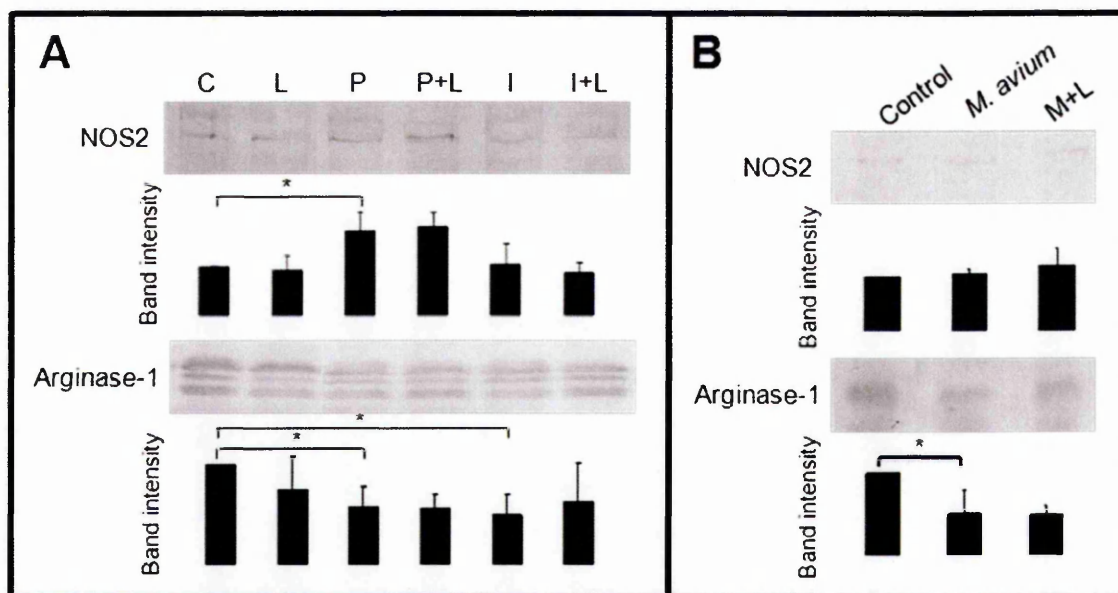


FIGURE 20. Effect of activation, *M. avium* infection and treatment with lapachol on L-arginine metabolism by THP-1 macrophages.

Macrophages were treated with P₃CSK₄ or IFN- γ , or infected with *M. avium* for 4 days, then cultured for 4 additional days in presence or not of lapachol. Cell lysates were electrophoresed, transferred to nitrocellulose membranes and western blotted, and the blotting membranes were probed with antibodies against the inducible enzymes nitric oxide synthase (iNOS) and arginase-1. Band intensities were determined using imageJ software.

A. Macrophages treated with P₃CSK₄ or IFN- γ , in association or not with lapachol (32 μ g/mL)

C: Control; L: lapachol; P: P₃CSK₄; P+L: P₃CSK₄ + lapachol; I: IFN- γ ; I+L: IFN- γ + lapachol

B. Macrophages infected with *M. avium*, then treated or not with lapachol (32 μ g/mL)

M+L : *M. avium* + lapachol

Results are expressed as means \pm S.E. (n=3, independent experiments). Significant differences ($p \leq 0.05$) in the comparisons indicated by brackets are shown by the symbol *.

5. Expression of pattern recognition, phagocytic and adhesion receptors

5.1. Expression of TLR2

TLRs play a major role among pattern recognition receptors (PRRs). They recognize pathogen-associated molecular patterns (PAMPS), which are molecules well represented in all pathogens. Basically, TLRs are able to trigger innate immune responses by identifying non-self-molecules. PAMPs include bacterial lipopolysaccharide (LPS), flagellin, lipoteichoic acid from Gram positive bacteria, peptidoglycans, surface carbohydrate-containing molecules, lipids and nucleic acid variants normally associated with viruses such as double-stranded RNA (dsRNA) or unmethylated CpG motifs. TLR2 forms heterodimers with TLR1 or TLR6, and this association allows the recognition of a broad

range of ligands. Recognition needs the engagement of auxiliary molecules such as CD14 (Carpenter and O'Neill 2007; Bowdish, Sakamoto et al. 2009). During the present work, the synthetic lipopeptide P₃CSK₄ was used as TLR2 ligand.

The expression of TLR2 by THP-1 macrophages was found to be significantly reduced by lapachol by about 50%, and the same effect was induced by P₃CSK₄ and *M. avium* infection (Fig. 21). These reductions can be interpreted as a cellular control to avoid over stimulation via this receptor. In the particular case of *M. avium*, the effect may represent an advantage, since TLR ligands trigger antimicrobial activity (Thoma-Uszynski, Stenger et al. 2001). No changes in surface TLR2 were induced by first line antibiotics.

5.2. Expression of CD14

CD14 is a 55 KDa glycoprotein found in the membrane of phagocytes. It works in association with TLRs in the recognition of bacterial LPS (Wright 1995). There is evidence that CD14 not only participates in cell activation by LPS but also mediates the phagocytosis of mycobacteria by macrophages, in association with other phagocytic receptors (Sendide, Reiner et al. 2005). CD14 also represents an important way of mycobacterium internalization by microglia (Peterson, Gekker et al. 1995). Thus, changes in the expression of CD14 can affect not only TLR-induced activations but also the phagocytosis of mycobacteria. CD14 expression is down-regulated upon macrophage differentiation (Daigneault, Preston et al. 2010).

The surface expression of CD14 was studied in THP-1 macrophages cultured in the presence of lapachol, P₃CSK₄ and during *M. avium* infection, as well as under the influence of first line antibiotics. Basal levels (resting cells) were very low, in agreement with Daigneault et al. (Daigneault, Preston et al. 2010). None of the cell treatments or infection produced significant changes in these low expression levels (Fig. 22).

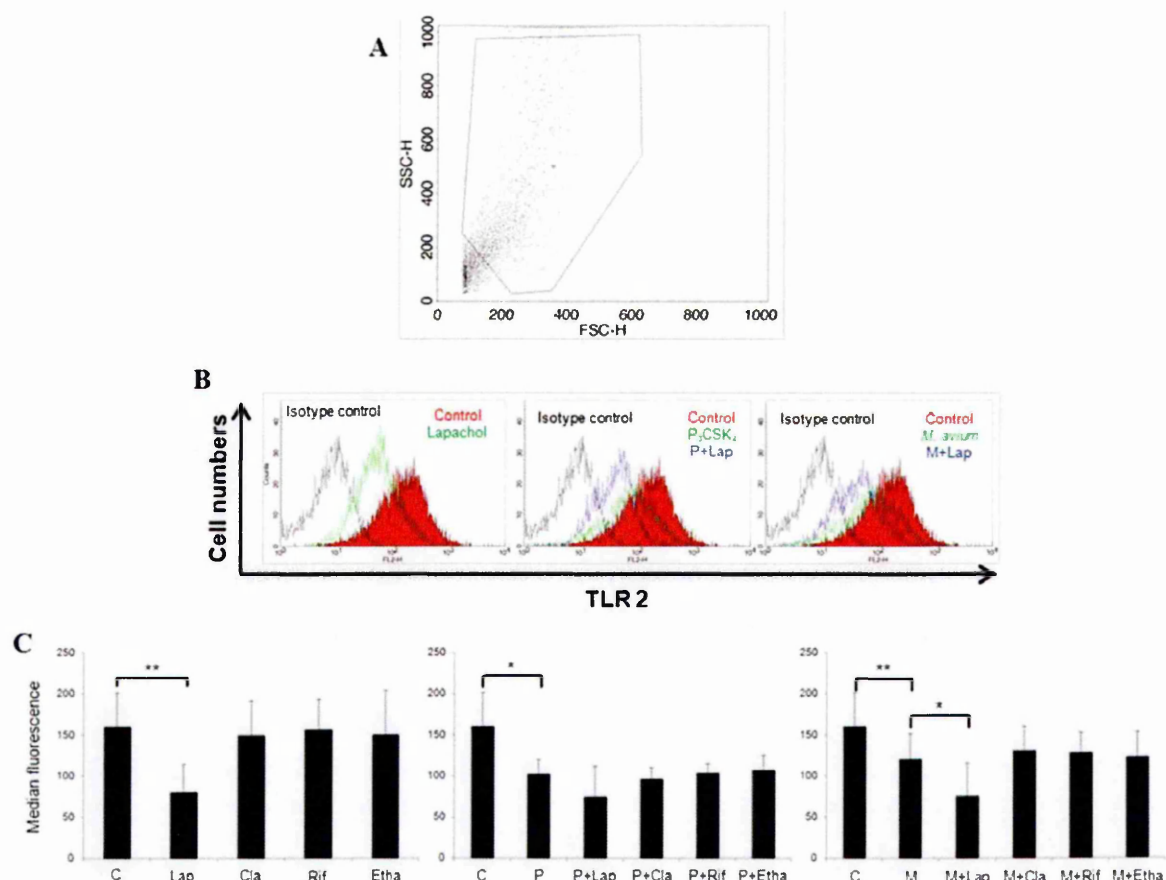


FIGURE 21. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface TLR2 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against TLR2 or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **B**.

B. Representative histograms of isotype controls and of TLR2 expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA. * $p \leq 0.05$; and ** $p \leq 0.01$.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin;

P+Rif: P₃CSK₄ +Rifampicin; P+Etha: P₃CSK₄ + Ethambutol;

M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;

M + Rif: *M. avium* + Rifampicin; M + Etha: *M. avium* + Ethambutol

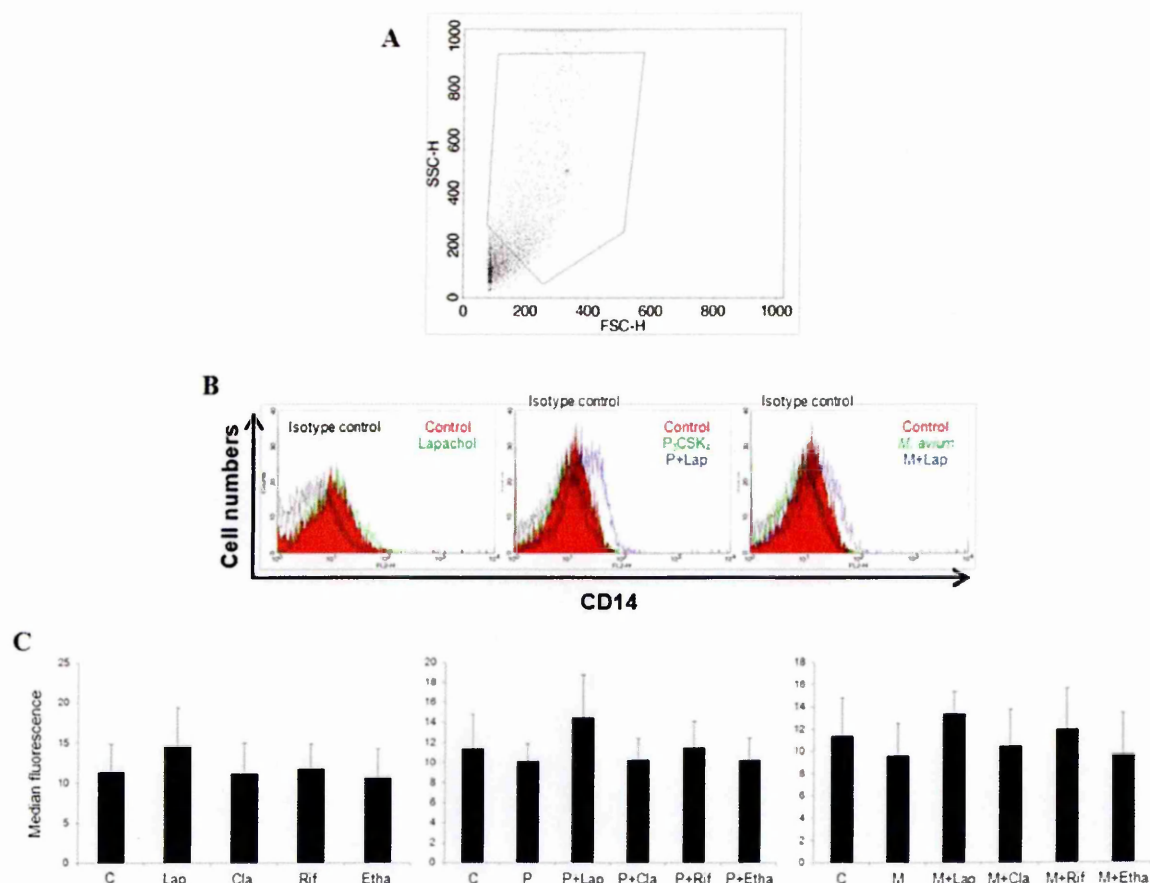


FIGURE 22. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface CD14 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against CD14 or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **B**.

B. Representative histograms of isotype controls and of CD14 expression in control, activated or infected or/and lapachol-treated cells, as indicated. The CD14 expression level was very low.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin;

P+Rif: P₃CSK₄+Rifampicin; P+Etha: P₃CSK₄+Ethambutol;

M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;

M + Rif: *M. avium* + Rifampicin; M + Etha: *M. avium* + Ethambutol

5.3. Expression of CD18

CD18 is the β subunit of β -integrins that associates with three different α subunits, namely CD11a (α_L), CD11b (α_M) and CD11c (α_X), to form LFA-1 (CD11a/CD18), Mac-1 or CR3 (CD11b/CD18) and CR4 (CD11c/CD18). Integrins can ligate iC3b, the adhesion molecule-1 (ICAM-1), bacterial products such as LPS, fibronectin, and coagulation factor X. These molecules are recognized by the integrin α -subunit (Petty, Worth et al. 2002). CR3 can also recognize mycobacterial antigens such as the antigen 85C (Hetland and Wiker 1994) and glycopeptidolipids (Irani and Maslow 2005), and is involved in the phagocytosis of opsonized and non-opsonized mycobacteria (Velasco-Velazquez, Barrera et al. 2003). Therefore, variations in the expression of CR3 can influence pathogen recognition and uptake.

The expression of CD18 was studied after TLR2 agonism, *M. avium* infection or treatment with first line antibiotics. It was significantly down-regulated by lapachol and *M. avium* infection (Fig. 23). A reduction of CD18 on the cell surface could result in a certain decrease in the rate of uptake of pathogens by macrophages and/or in cell adhesion.

5.4. Expression of Fc receptors

Fc receptors (Fc γ R) are a family of surface molecules that bind to IgG-opsonised pathogens by recognizing the Fc portion of IgG. They belong to three different groups: Fc γ RI or CD64, Fc γ RII or CD32 and Fc γ RIII or CD16, all of them being expressed on macrophages. Fc γ RI binds monomeric IgG with high-affinity, while Fc γ RII and III recognize only multimeric immune complexes and have, therefore, been named low-affinity receptors (Dijstelbloem, van de Winkel et al. 2001). Fc receptors promote microorganism internalization, an efficient antigen presentation, and can trigger oxidative stress (Amigorena and Bonnerot 1999; Park 2003). Binding to these receptors can generate immune response stimulatory or inhibitory signals (Gerber and Mosser 2001).

The expression of FcγRI (CD64) was not affected by any of the treatments (Fig. 24). FcγRII (CD32) expression was significantly down-regulated by lapachol, TLR2 agonism and *M. avium* infection (Fig. 25). FcγRIII (CD16) was not significantly affected by any of the treatments (Fig. 26). In brief, lapachol only reduced the expression of FcγRII, an effect produced also by TLR2 agonism and *M. avium* infection. Reductions in FcγRII could cause a decreased up-take of pathogens but the fact that the high affinity FcγRI was not altered suggests that phagocytosis of IgG-opsonised organisms might even be unaffected.

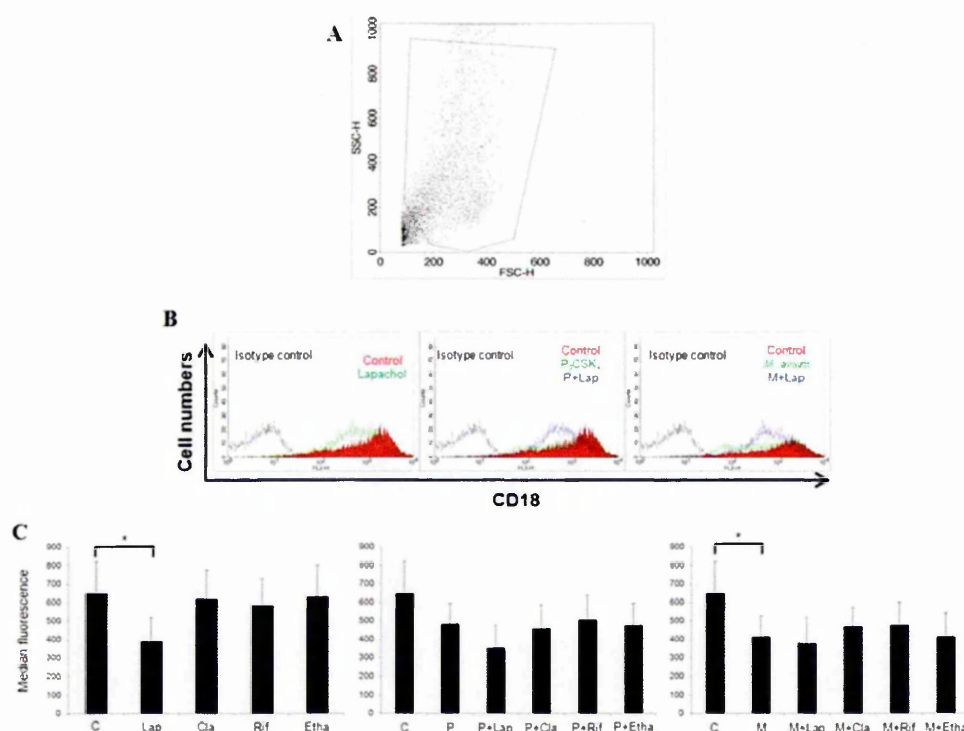


FIGURE 23. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface CD18 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against CD18 or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **B**.

B. Representative histograms of isotype controls and of CD18 expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA. * p ≤ 0.05.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin;

P+Rif: P₃CSK₄+Rifampicin; P+Etha: P₃CSK₄+Ethambutol;

M: *M. avium*; M+L: *M. avium*+Lapachol; M+Cla: *M. avium*+Clarithromycin;

M+Rif: *M. avium*+Rifampicin; M+Etha: *M. avium*+Ethambutol

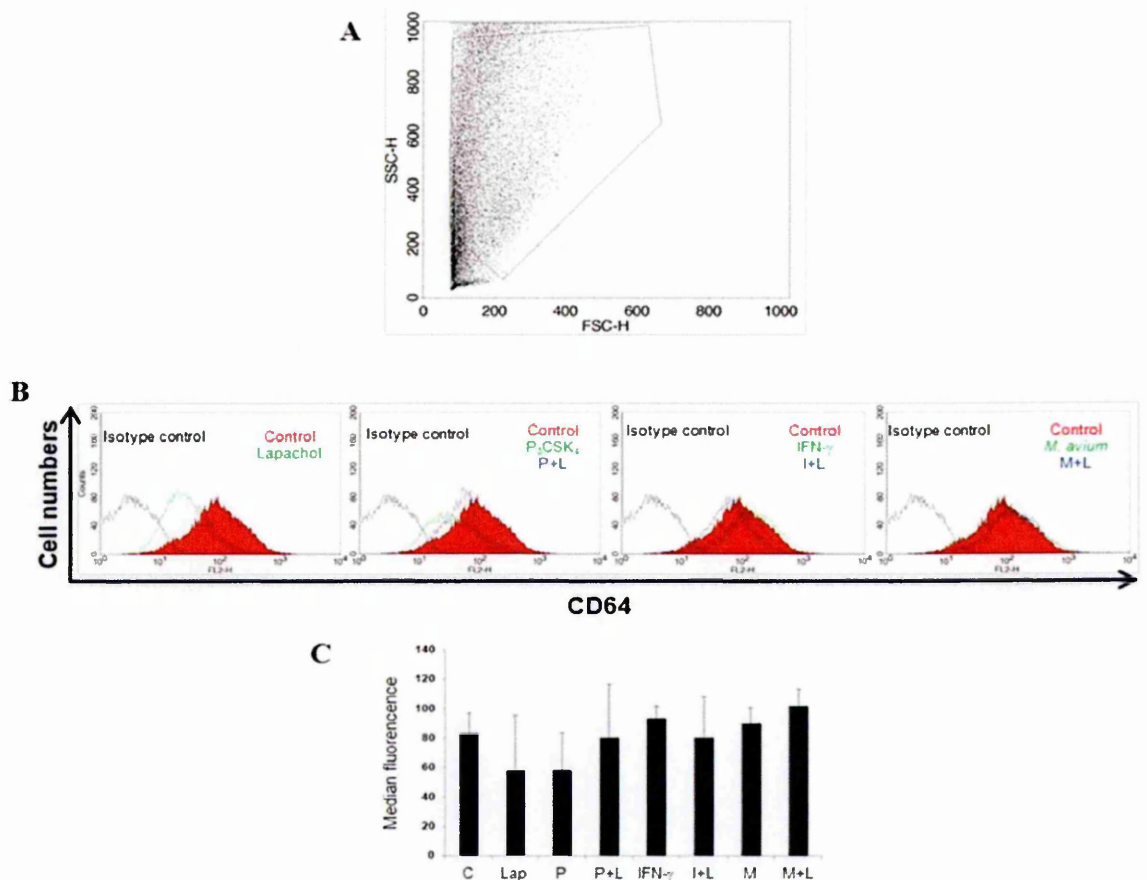


FIGURE 24. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface CD64 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 μ g/mL lapachol, 0.56 μ g/mL clarithromycin, 1.6 μ g/mL rifampicin or 0.35 μ g/mL ethambutol. Cells were stained with PE-conjugated mAb against CD64 or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **B**.

B. Representative histograms of isotype controls and of CD64 expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means \pm S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄ + lapachol; P+Cla: P₃CSK₄ + Clarithromycin;

P+Rif: P₃CSK₄ + Rifampicin; P+Etha: P₃CSK₄ + Ethambutol;

M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;

M + Rif: *M. avium* + Rifampicin; M + Etha: *M. avium* + Ethambutol

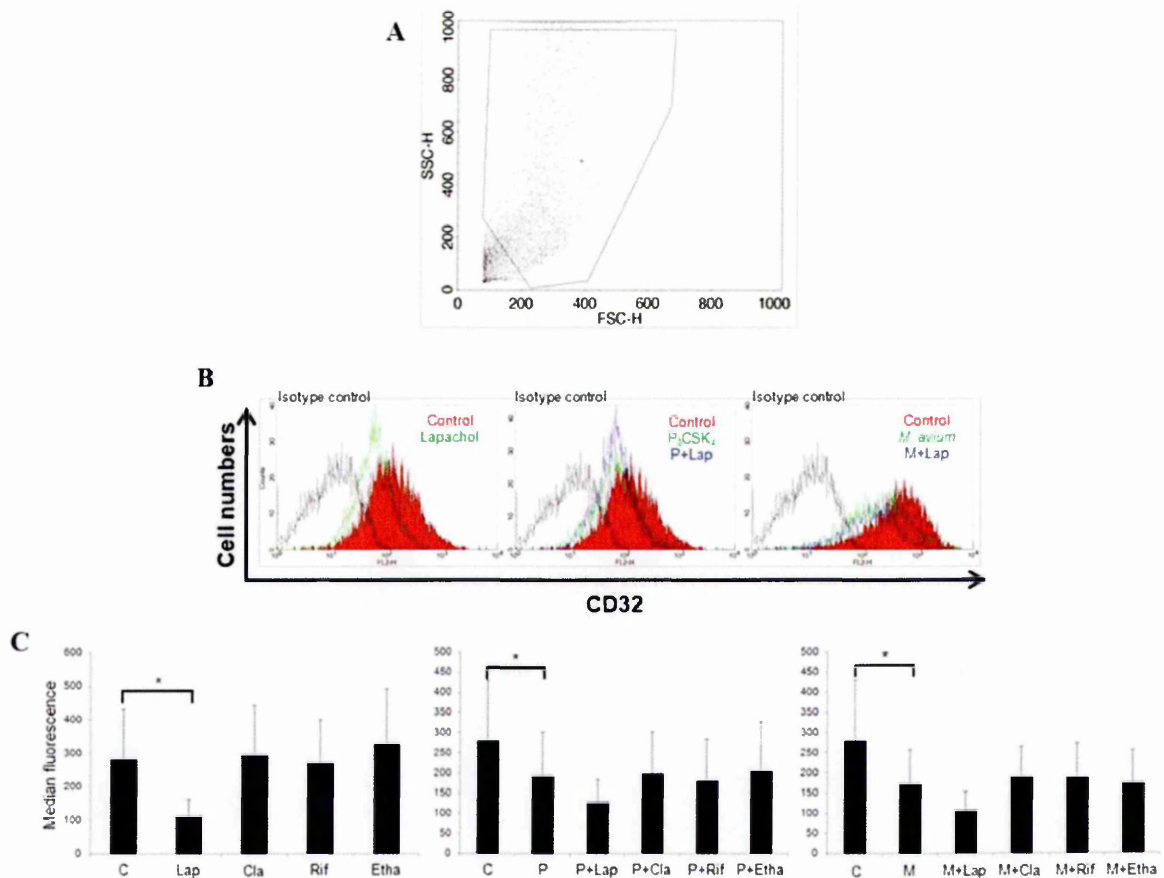


FIGURE 25. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface CD32 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against CD32 or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **B**.

B. Representative histograms of isotype controls and of CD32 expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA. * p ≤ 0.05.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄ + lapachol; P+Cla: P₃CSK₄ + Clarithromycin;

P+Rif: P₃CSK₄ + Rifampicin; P+Etha: P₃CSK₄ + Ethambutol;

M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;

M + Rif: *M. avium* + Rifampicin; M + Etha: *M. avium* + Ethambutol

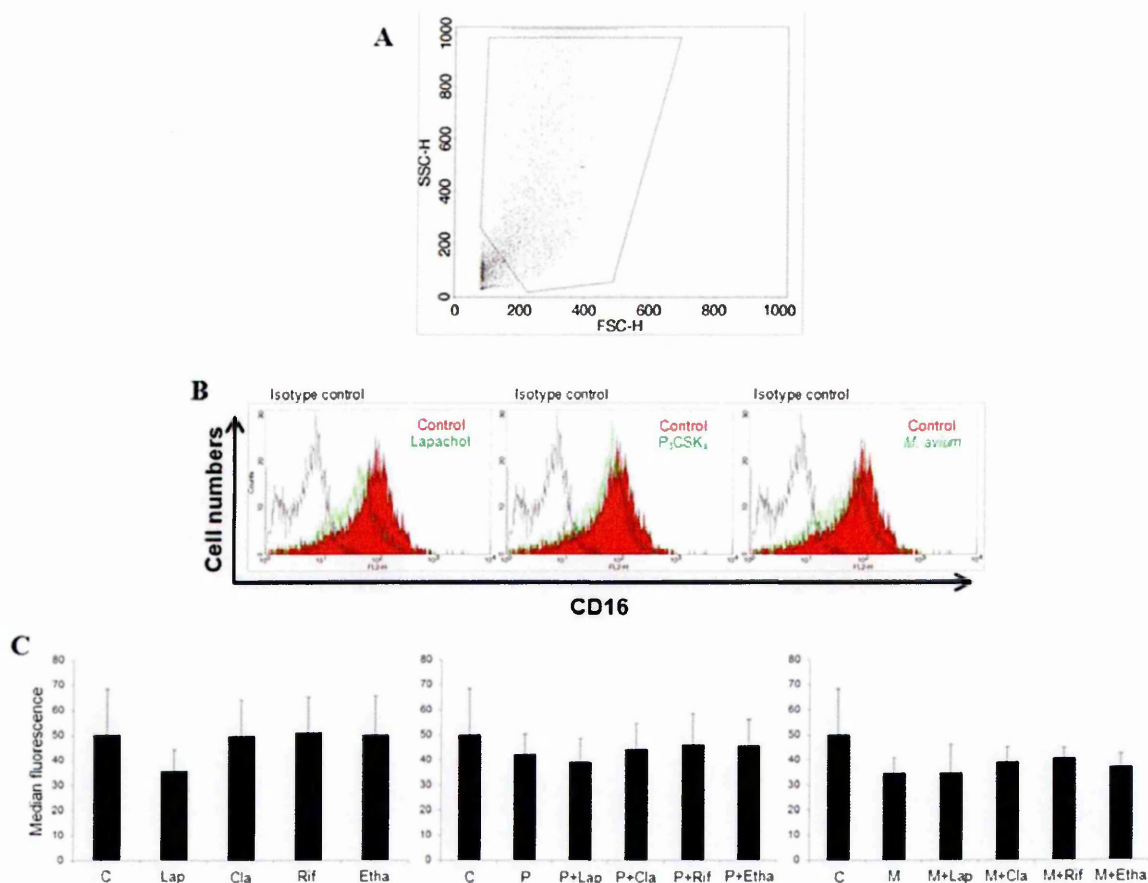


FIGURE 26. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface CD16 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against CD16 or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **B**.

B. Representative histograms of isotype controls and of CD16 expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin;

P+Rif: P₃CSK₄+Rifampicin; P+Etha: P₃CSK₄+Ethambutol;

M: *M. avium*; M+L: *M. avium*+Lapachol; M+Cla: *M. avium*+Clarithromycin;

M+Rif: *M. avium*+Rifampicin; M+Etha: *M. avium*+Ethambutol

5.5. Expression of Mannose Receptor (ManR)

ManR is a C-type lectin receptor that recognizes mannose-containing carbohydrates in a Ca^{++} -dependent manner. ManR can promote the phagocytosis of a wide variety of pathogens, such as yeasts, virus and bacteria (Linehan, Martinez-Pomares et al. 2000; Willment and Brown 2008). It specifically recognizes as a ligand the mycobacterial cell wall component lipoarabinomannan (Schlesinger, Hull et al. 1994), thus promoting the internalization of these pathogens as well as antigen presentation (Amigorena and Bonnerot 1999).

The expression of ManR by THP-1 cells was significantly down-regulated by lapachol, *M. avium* infection and the association of infection and lapachol, as shown in Fig. 27. These changes could compromise to some extent the internalization of yeasts and bacteria through ManR.

5.6. Expression of Scavenger Receptor AI (SR-AI)

SR-A receptors bind to modified oxidised or acetylated low density lipoproteins and a vast list of ligands including polyribonucleotides, polysaccharides and lipids. Trehalose dimycolate is a cell wall component of *M. tuberculosis* that binds to macrophage SR-A and this results in phagocytosis. SR-A is involved in the initiation of innate and acquired immune responses, and also plays a protective role against excessive responses (Gough and Gordon 2000; Palecanda and Kobzik 2001; Peiser and Gordon 2001).

The expression of SR-AI in THP-1 macrophages was not significantly affected by any treatment nor by *M. avium* infection (Fig. 28), hence recognition and internalization via SR-AI is not compromised.

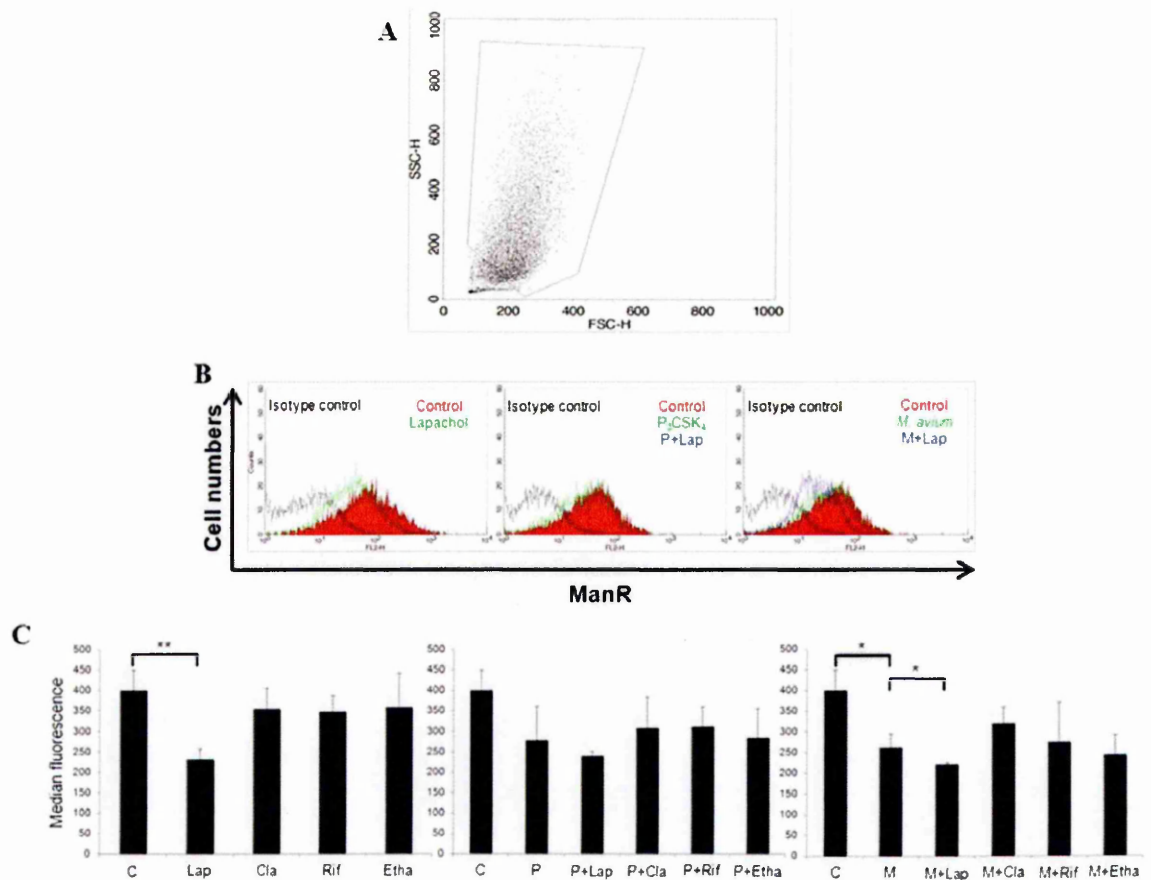


FIGURE 27. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface mannose receptor (ManR) by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against ManR or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **B**.

B. Representative histograms of isotype controls and of ManR expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA. * $p \leq 0.05$; and ** $p \leq 0.01$.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin;

P+Rif: P₃CSK₄+Rifampicin; P+Eth: P₃CSK₄ + Ethambutol;

M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;

M + Rif: *M. avium* + Rifampicin; M + Eth: *M. avium* + Ethambutol

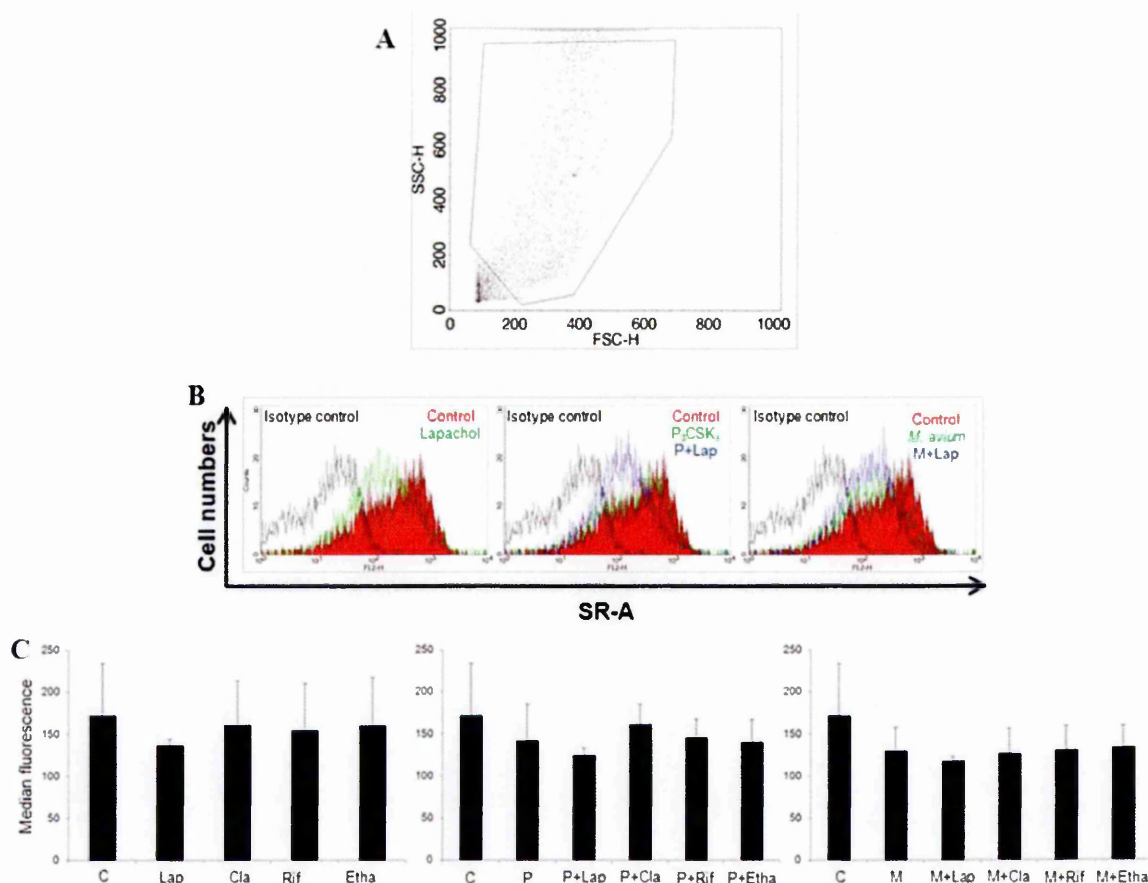


FIGURE 28. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface Scavenger Receptor-A (SR-A) by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with FITC-conjugated swine Ab against SR-A or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **B**.

B. Representative histograms of isotype controls and of SR-A expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin;

P+Rif: P₃CSK₄+Rifampicin; P+Eth: P₃CSK₄ + Ethambutol;

M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;

M + Rif : *M. avium* + Rifampicin; M + Etha: *M. avium* + Ethambutol

5.7. Expression of CD87 (uPAR)

Urokinase-type plasminogen receptor (uPAR or CD87), is widely expressed and plays an important role in cellular migration. This receptor binds urokinase-type plasminogen (uPA) on the cell surface, and uPA converts plasminogen in plasmin. Plasmin activates metalloproteinases, the proteolytic action of which allows cellular penetration within tissues (Murphy, Stanton et al. 1999). There is also evidence that proteolysis is not the sole way by which uPAR can control cell migration (Ge and Elghetany 2003).

The expression of CD87 was studied in resting, TLR2-agonised and *M. avium*-infected macrophages, treated or not with lapachol or first line antibiotics. Significant reductions in CD87 levels were only produced by P₃CSK₄ treatment associated with lapachol, mycobacterial infection and treatment with lapachol (Fig. 29). CD87 decreases could compromise the ability of macrophages to migrate to infection foci.

6. Phagocytosis

The fact that lapachol and TLR2 ligation down-regulated the surface expression of some phagocytic receptors prompted studies on the phagocytosis ability of macrophages. Macrophages were treated with P₃CSK₄ for 4 days and then treated or not with lapachol for 4 additional days. At this point cells were mixed with Gram-positive or Gram-negative organisms, mycobacteria or inert particles and uptake was measured. Analysis was by microscopy, FACS or colony counting, as indicated. Binding at 4°C was determined as a control of attachment to the cell surface as opposed to the attachment followed by ingestion that takes place at 37°C.

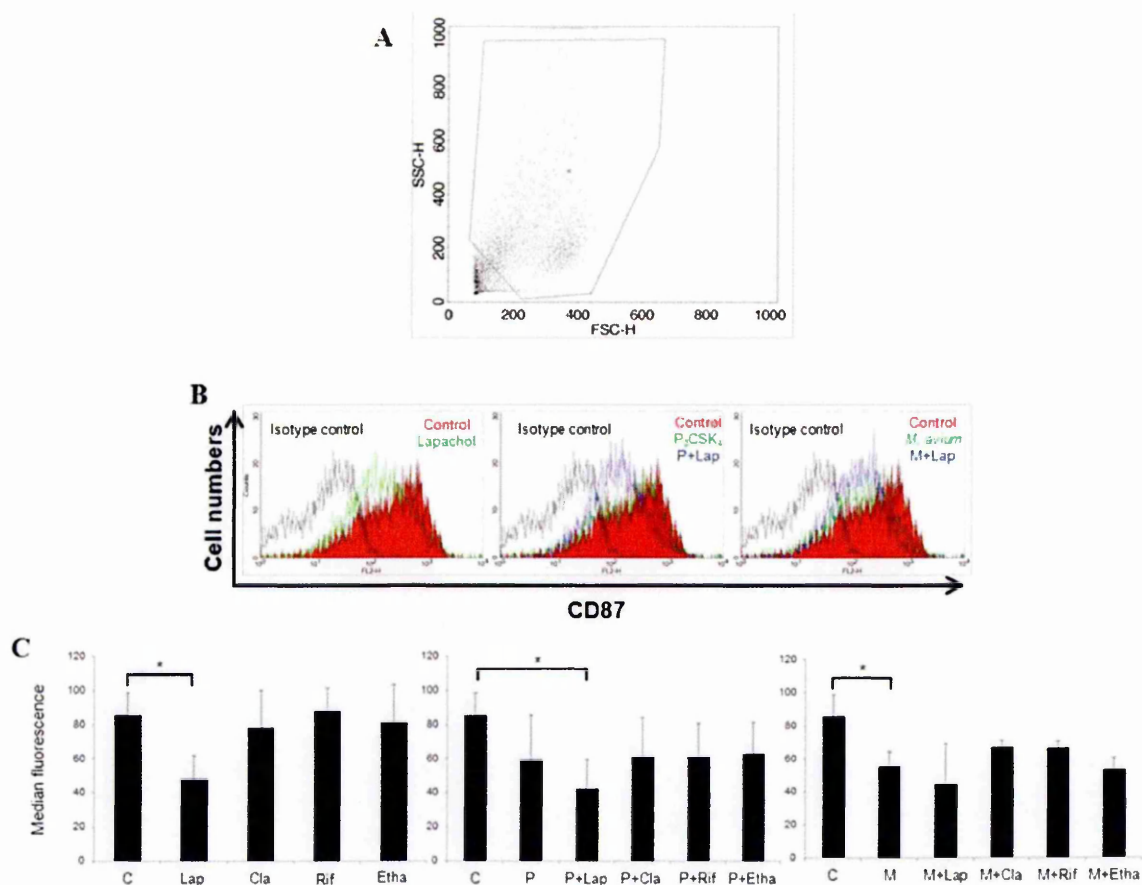


FIGURE 29. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of CD87 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against CD87 or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **B**.

B. Representative histograms of isotype controls and of CD87 expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA. * $p \leq 0.05$.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin;

P+Rif: P₃CSK₄+Rifampicin; P+Etha: P₃CSK₄ + Ethambutol;

M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;

M + Rif: *M. avium* + Rifampicin; M + Etha: *M. avium* + Ethambutol

6.1. Latex beads

When analyzing mean fluorescence intensities (Fig.30, C: Phagocytic activity plot), both binding and internalization of fluorescent latex beads was decreased ~40% by lapachol in resting macrophages, as well as in TLR2-agonised cells. Nevertheless, analysis of the percentage of cells that had ingested any number of latex particles (Fig. 30, C: % Phagocytosis plot) did not show significant variations among the different cell treatments. This can be explained by a decreased uptake rate but not complete inhibition of ingestion under the influence of lapachol, which results in cells containing fewer particles each and therefore exhibiting a lower fluorescence.

6.2. *S. aureus*

The binding and internalization of these gram-positive bacteria by resting cells was not significantly affected by lapachol (Fig. 30, A). TLR2 agonism resulted in an increase of both bound and internalized bacteria, with the latter being inhibited by treatment of TLR2-agonised cells with lapachol. Inhibition of internalization but not binding suggests a slowing down of the rate of microorganism uptake.

6.3. *S. typhimurium*

Lapachol diminished the number of resting but not TLR2-agonised cells that internalize this pathogen (Fig. 30, C: % Phagocytosis plot). However, the number of bacteria bound or internalized was not significantly affected by lapachol or any other treatment (Fig. 30, C: Phagocytic activity plot), which suggests that the uptake rate was not altered or was even increased by lapachol in this case.

6.4. *M. avium*

The binding of *M. avium* to macrophages was not affected in any condition (Fig. 30, C: Phagocytic activity plot). Instead, internalization by either resting or TLR2-agonised macrophages was sensibly decreased (50-80%), although not suppressed, by lapachol (Fig. 30, C: Phagocytic activity plot). Since the amount of cells that were able to phagocytose fluorescent *M. avium* was not significantly affected by lapachol or any other treatment (Fig. 30, C: % Phagocytosis plot), a decrease in the rate of uptake is likely to be occurring. The FACS results shown in Fig. 30 regarding *M. avium* phagocytosis were in agreement with those obtained by determining directly the number of intra-cellular bacteria by fluorescence microscopy, shown in Fig. 31.

The reduction in the uptake rate of *M. avium* and *S. aureus* caused by lapachol, without bacterial binding to cells being affected, is likely to be due to some signaling or cytoskeleton-related interference and not to a receptor binding defect.

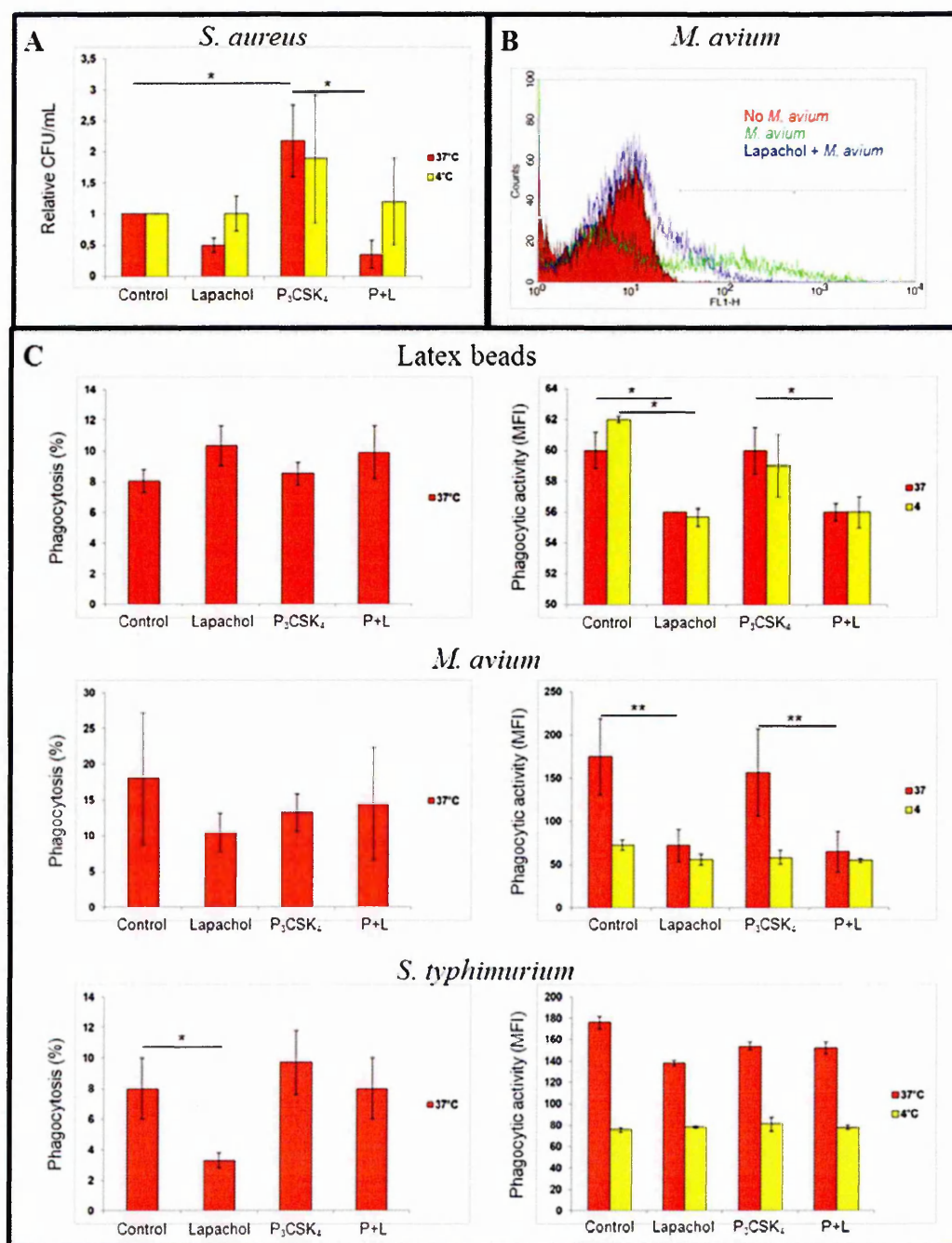


FIGURE 30. Influence of TLR2 agonism and lapachol on the binding and phagocytosis of latex beads, *M. avium*, *S. aureus* and *S. typhimurium*

Macrophages were cultured with 300nM P₃CSK₄ for 4 days and then treated or not with lapachol (32µg/mL) for 4 additional days. They were then incubated at 4°C or 37°C with FITC-*M. avium*, for 4 hours, or with *S. aureus*, GFP-*S. typhimurium* or FITC-latex beads, for 30 minutes. Cell-associated bacteria/particles were determined after washing excess bacteria/particles with PBS.

A. *S. aureus* phagocytosis: Cells were lysed and plated on Luria agar for colony counting. Results are expressed as relative CFU values, taking as 1 for each experiment the value of cell-associated CFUs corresponding to untreated macrophages. Means ± S.E. (n=3, independent experiments). **B.** Representative FACS histograms of a *M. avium* phagocytosis experiment. **C.** Phagocytosis (%) and phagocytic activity of fluorescent latex beads, *M. avium*, and *S. typhimurium*. Phagocytosis (%) is expressed as [number of cells containing any number of particles]/[total gated cell number] X 100. Phagocytic activities are expressed as the median fluorescence intensities (MFI) of the FACS histograms (means ± S.E., n=3, independent experiments). Statistical differences were analysed by ANOVA (* p ≤ 0.05, ** p ≤ 0.01).

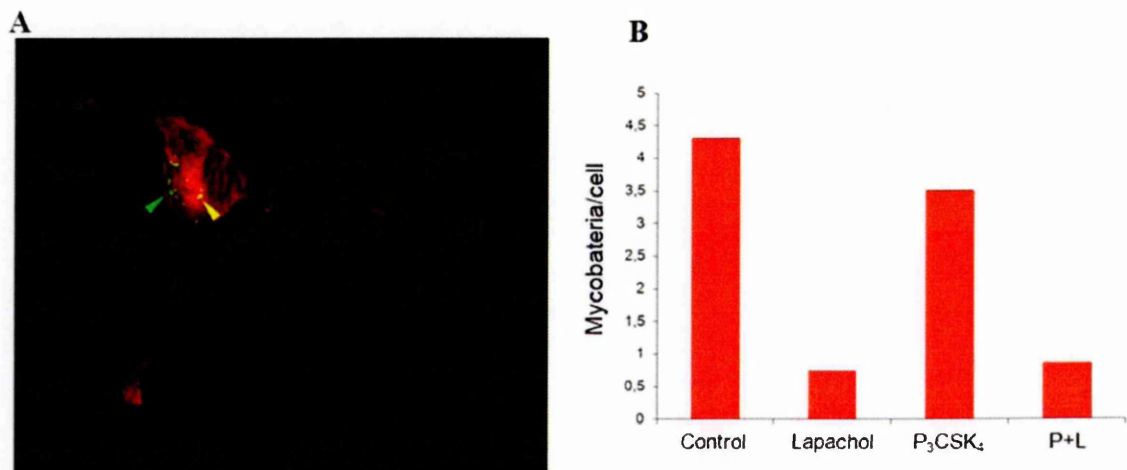


FIGURE 31. Phagocytosis of *M. avium* by resting, activated and/or lapachol-treated THP-1 macrophages, evaluated by fluorescence microscopy.

Macrophages were incubated with 300nM P₃CSK₄ for 4 days and then treated or not with lapachol (32μg/mL) for 4 additional days. Cells were then incubated at 37°C with *M. avium*-FITC for 4 hours to allow internalization. They were washed 3 times to remove extracellular mycobacteria, fixed on glass slides with paraformaldehyde and reacted with phalloidin for actin counterstaining. The number of internalized bacteria was counted in 100 cells.

A. Illustrative picture showing phagocytosed (yellow arrow) and surface (green arrow) *M. avium*.

B. Bar graph of the ratios between internalized bacteria and cells observed in control, lapachol-treated and/or TLR2-agonised macrophages.

P+L : P₃CSK₄ + lapachol.

7. Secretion of pro- and anti-inflammatory cytokines

Tissue invasion by pathogens generates inflammation and this is orchestrated by a number of factors including cytokines. Cytokines can have pro- or anti-inflammatory effects, and the balance between them controls the immune response and minimizes tissue damage (Opal and DePalo 2000). Cytokines can be divided in two main groups according to their involvement in acute inflammation or in chronic inflammation. Acute inflammation is associated to pro-inflammatory cytokines and/or chemoattractants, and

chronic inflammation is characterized by the secretion of anti-inflammatory cytokines mediating tolerance (Feghali and Wright 1997).

The expression of acute cytokines and anti-microbial proteins is regulated by nuclear factor- κ B (NF- κ B). NF- κ B becomes activated after PAMP recognition by PRRs or by pro-inflammatory cytokines and plays a crucial role in the regulation of innate and adaptive immune response (Li and Verma 2002).

Some natural compounds have been shown to exhibit immunomodulatory effects. For instance, kavain present in root extracts of *Piper methysticum* prevents the secretion of the pro-inflammatory cytokine TNF- α by LPS-stimulated THP-1 cells (Pollastri, Whitty et al. 2009). Plumbagin, a naphthoquinone found in plants, induces an anti-inflammatory effect by inhibiting the secretion of IL-2, IL-4, IL-6 and IFN- γ by stimulated mouse lymphocytes through preventing the degradation of I κ B and the subsequent NF- κ B activation (Checker, Sharma et al. 2009).

Lapachol, the object of the present work, is a naphthoquinone of the same family as plumbagin, and its effects on cytokine secretion or NF- κ B activation have not been studied yet. Here, the effect of lapachol on the secretion of pro- and anti-inflammatory cytokines by P₃CSK₄- or IFN- γ -treated macrophages or *M. avium*-infected macrophages is shown.

7.1. TNF- α

TNF- α secretion is shown in Fig. 32. Lapachol suppressed the basal secretion of TNF- α by resting and IFN- γ -treated macrophages, but did not affect the significant up-regulation produced by TLR2 agonism. The increase in TNF- α secretion triggered by *M. avium* infection was reduced but not suppressed by treatment with lapachol. This reduction could be a consequence of the direct effect of lapachol on the viability of intracellular mycobacteria.

7.2. IL-1 β

The secretion of IL-1 β , another pro-inflammatory cytokine, was significantly reduced (40-50%) by lapachol in resting and IFN- γ -treated cells (Fig. 32). No other significant change was observed.

7.3. IL-10

IL-10 is an anti-inflammatory cytokine known to be secreted by *M. avium*-infected cells (Bermudez and Champs 1993; Reiling, Blumenthal et al. 2001). A significant, pronounced inhibitory effect of lapachol on IL-10 secretion was observed in resting, TLR2-agonised, IFN- γ -treated and *M. avium*-infected cells (Fig. 32). IL-10 opposes the effects of pro-inflammatory cytokines such as IFN- γ and TNF- α , therefore a reduced secretion of IL-10 is expected to result in an enhanced bacterial killing capacity of macrophages. In the case of *M. avium*-infected cells, treatment with lapachol would favour the intra-cellular killing of those microorganisms due to the curbing of IL-10 secretion (Roach, Barton et al. 1995). Since the secretion of TNF- α and IL-1 β elicited by TLR2 agonism was not significantly affected by lapachol, the additional reduction in IL-10 levels would create a scenario favouring the control of *M. avium* intracellular growth in macrophages.

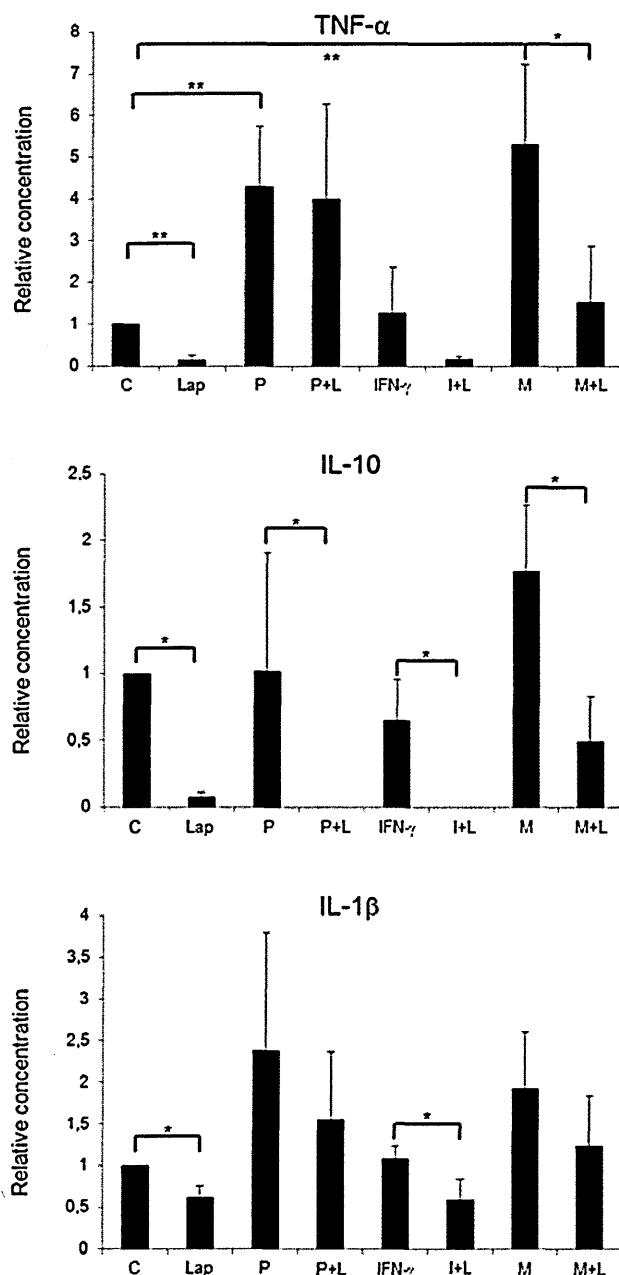


FIGURE 32. Cytokine secretion by resting, lapachol treated, TLR2-agonised, IFN-γ-treated and *M. avium*-infected THP-1 macrophages.

Resting (control) cells or cells activated with IFN-γ or P₃CSK₄ or infected with *M. avium* for 4 days were treated with lapachol (32μg/mL) for 4 additional days. Cell culture supernatants were used to determine TNF-α, IL-10 and IL-1β concentrations by ELISA.

The data shown are means ± S.E. (n=3, independent experiments), taking as 1 the control value (untreated cells) for each of the experiments. The absolute values of the controls were (ranges, n=3): 20 to 80 pg/mL for TNF-α, 9 to 22 pg/mL for IL-10 and 56 to 128 pg/mL for IL-1β. Statistical analysis was performed by ANOVA. Significant differences are indicated by star symbols (* p ≤ 0.05; ** p ≤ 0.01).

C: Control; Lap: lapachol; P: P₃CSK₄; P + L: P₃CSK₄+ lapachol;

I + L: IFN-γ + lapachol; M: *M. avium*; M+L: *M. avium* + lapachol

ER is a compartment of the secretory pathway that is responsible for the synthesis, modification, and delivery of proteins. The flux of protein synthesis and folding is controlled in this cellular location, and is disturbed when the influx of nascent, unfolded proteins chains exceeds the folding capacity of the ER. This generates ER stress, followed by the unfolded protein response (UPR) during which chaperones are up-regulated, since they are the molecules responsible to fold and unfold proteins (Schroder and Kaufman 2005). In the present study we found that neither lapachol nor AC201 increased the levels of grp78, therefore their presence did not perturb ER function and homeostasis. Surprisingly, TLR2 agonism reduced the expression of grp78. It is known that TLR agonism can induce autophagy via its adaptor protein MyD88 and TRIF (Delgado, Elmaoued et al. 2008; Shi and Kehrl 2008). A reduction in grp78 levels could affect autophagy, since grp78 is required for autophagosome formation (Li, Ni et al. 2008).

Pathogen sensing by innate cells is mediated by PRRs, which constitute immune surveillance detectors by recognizing PAMPs present in pathogens. Among PRRs, toll-like receptors (TLRs) are responsible for the detection of various classes of pathogens and molecules derived therefrom. The ligation of PAMPs to TLRs activates a signal transduction cascade leading to the activation of the transcription factor NF- κ B, which regulates the expression of pro-inflammatory cytokines such TNF- α , IL-1 β and IL-12 that activate the immune response and enhance the killing ability of phagocytes by inducing the generation of oxygen active species (Henneke, Takeuchi et al. 2002) and nitrogen metabolites (Thoma-Uszynski, Stenger et al. 2001). The protective role of TLRs is demonstrated by the failure of TLR2 knockout mice to increase the levels of TNF- α and IL-12 after infection with *M. tuberculosis* (Drennan, Nicolle et al. 2004), with the result of an increased susceptibility to this disease. TLR2 deficiency also enhances the susceptibility to pneumonic tularemia (Malik, Bakshi et al. 2006). Moreover, polymorphisms in human TLR2 are associated with an increased susceptibility to tuberculous meningitis (Thuong,

Hawn et al. 2007), and allergy-induced TLR2 down-regulation contributes to decreased mycoplasma clearance in mice (Wu, Martin et al. 2008).

During the present work, THP-1 macrophages responded to TLR2 agonism and infection with *M. avium* by secreting significant amounts of TNF- α (Fig. 32). The compound here being studied, lapachol, did not interfere with the TNF- α response since it did not affect the TLR2- elicited secretion of this cytokine. In the case of *M. avium*-infected macrophages, lapachol inhibited the secretion of TNF- α to levels similar to those of resting cells. This is likely to be a consequence of the fact that lapachol is curbing the intracellular growth of the pathogen, therefore reducing the extent of TNF- α secretion by disappearance (degradation) of stimulating molecules from the bacterial surface. Lapachol treatment did result in a partial inhibition of IL-1 β secretion by resting and IFN- γ -treated macrophages (Fig. 32). Infection of macrophages with *M. avium* induces the secretion of the immunosuppressive, anti-inflammatory cytokine IL-10, which favours pathogen survival (Grutz 2005). The role of IL-10 in the resistance of *M. avium* to innate immune responses was highlighted by Murray et al (Murray and Young 1999), who observed that IL-10 deficient mice respond more efficiently to *M. avium* infections. The THP-1 macrophages here used responded to *M. avium* infection with a non-significant increase in IL-10 levels, which were significantly reduced to basal values when the infected cells were treated with lapachol (Fig. 32). The general effect of lapachol, which suppressed even basal levels of IL-10, is in favour of a pro-inflammatory response and against mycobacterial survival.

The levels of expression TLR2 on phagocytic cells could regulate the level of cell response to stimuli. Mae et al (Mae, Iyori et al. 2007) demonstrated that TLR2 ligands do not change the expression of this receptor on THP-1 monocytes. Instead, Wang et al (Wang, Lafuse et al. 2000) detected increased levels of TLR2 mRNA after *M. avium* infection of mouse macrophages. The results here reported, using THP-1 macrophages,

show that TLR2 agonism, as well as *M. avium* infection and treatment with lapachol, influenced negatively the expression of TLR2 by these cells (Fig. 21). The relatively modest down-regulation of TLR2 expression observed could represent a mechanism to avoid the over-activation of the PMA-treated THP-1 macrophages. In spite of the reduction in surface TLR2, many TLR2 agonism-mediated events were not affected by lapachol, namely increases NOS2 (Fig. 20), MnSOD (Fig. 19), p47^{phox} (Fig. 18) and TNF- α secretion (Fig. 32); decrease in arginase-1 levels (Fig. 20). This suggests that the lapachol-induced decrease in surface TLR2 probably does not result in impairments of TLR2-mediated functions.

The present study has shown that stimulation of THP-1 macrophage-like cells with P₃CSK₄ or *M. avium* infection, or treatment with lapachol, often induced a partial down-regulation of the expression of surface receptors involved in bacterial internalization, including mycobacteria (Figs. 23 and 25). Some of these receptors can be also involved in cell migration (CD87, Fig. 29). Mannose receptor (ManR) but not scavenger receptor AI (SR-AI) expression was down-regulated by the TLR2 agonist P₃CSK₄ (Fig. 27 & 28). These results do not agree with those of Mae et al., who found an up-regulation of the mRNA levels of SR-AI and some C-type lectins. The differences can be due to the fact that Mae et al. have used non-differentiated THP-1 cells instead of inducing differentiation to macrophage-like cells with 20 nM PMA as performed during the present work. Besides, results based on mRNA levels often do not coincide with those from direct measurements of protein expression.

Mycobacteria can be opsonically or non-opsonically internalized after binding to a wide range of receptors. TLRs can regulate phagocytosis by activation of phagocytic function genes (Doyle, O'Connell et al. 2004). Since the results here shown indicate that TLR agonism and lapachol down-regulate to some extent the expression of some receptors involved in phagocytosis, it was important to determine whether particle/bacterial

internalization was affected. However, it must be pointed that phagocytosis can also be altered by cytoskeleton-related and energy metabolism events and not just by variations in phagocytosis receptor levels. Mae et al. (Mae, Iyori et al. 2007) reported that TLR2 agonism by the diacylated lipopeptide FSL-1 enhanced *S. aureus* and *E. coli* phagocytosis, but did not influence the internalization of latex beads by non-differentiated THP-1 cells. In the present work, THP-1 macrophage-like cells were treated with the triacylated lipopeptide P₃CSK₄ and this significantly increased the internalization of *S. aureus* but not the ingestion of latex beads, *M. avium* or *S. typhimurium* (Fig. 30). Lapachol reduced significantly the amount of ingested *M. avium* and latex, but not that of *S. aureus*. It is worth noting that even when lapachol inhibited internalization measured as the total number of particles ingested, it did not have an effect on the % of phagocytosis except for a decrease observed in the case of *S. typhimurium*. The % of phagocytosis was defined as the proportion of cells that have ingested any number of particles, i.e. independently of the amount ingested. The situation after treatment with lapachol is one where the number of macrophages that are actively phagocytosing is the same as for untreated cells, but lapachol-treated cells contain on average fewer particles/bacteria per cell than those untreated. In the case of *S. typhimurium*, there seems to be fewer phagocytosing macrophages under the influence of lapachol. The diminished internalizations observed in the presence of lapachol are not likely to have been caused by down-regulations of phagocytic receptor expression, since no significant differences in surface binding at 4°C were detected (Fig. 30). Phagocytosis is a complex event involving a continuous rearrangement of actin filaments (Aderem and Underhill 1999; Ernst 2000; May and Machesky 2001). Therefore, impairments in the efficiency of cytoskeleton dynamics or in the signaling leading to it could be the cause of the partially diminished phagocytosis produced by lapachol. The consequences of a lower internalization rate in a physiological context are difficult to predict. They do not necessarily have to result in a higher incidence of infection, since the ratios macrophages/bacteria *in vivo* are much higher than those in *in*

vitro experiments. A reduced ability of each individual macrophage to ingest microorganisms could mean a greater number of pathogen ingesting cells, each containing a smaller number of microorganisms. Besides, particle internalization by other important cells of the first line of defense (e.g. neutrophils and DCs), if not affected or less affected by lapachol, could take care of the clearance of many bacteria. This would diminish the impact of a reduced phagocytosis by macrophages. To conclude, even with lapachol causing a diminished rate of *M. avium* internalization, the ability of the naphthoquinone to arrest and control intracellular *M. avium* growth was still satisfactory.

Chemotaxis and cell adhesion are important events involved in cell migration from the bloodstream to tissues. Therefore, the expression of receptors involved in these phenomena was examined. Cell surface CD87 (uPAR) was down-regulated by lapachol in resting cells, by TLR2-agonism in association with lapachol and by *M. avium* infection (Fig. 29). Aung et al (Aung, Wu et al. 2005) reported the up-regulation of the expression of uPAR in blood monocytes but not in alveolar macrophages after *M. tuberculosis* infection. The up-regulation is associated with TGF- β activity. Juffermans et al (Juffermans, Dekkers et al. 2001) reported a higher expression of this receptor on monocytes from *M. tuberculosis* infected patients. The down-regulations in uPAR expression observed in the present work could be explained by the fact that THP-1 macrophages are different from blood monocytes and by the use of *M. avium* and not *M. tuberculosis*.

ICAM-1 (CD54), another receptor involved in cell adhesion, binds to integrins such as LFA-1 and Mac-1, playing an important function as co-stimulator molecule during the formation of immunological synapses. Its expression was not affected by IFN- γ treatment (Fig. 35). The up-regulation of CD54 expression is associated with the effect of the pro-inflammatory cytokines IFN- γ and TNF- α (Roebuck and Finnegan 1999), whereas the anti-inflammatory cytokine IL-10 and *M. tuberculosis* infection down-regulate its expression in macrophages (de la Barrera, Aleman et al. 2004).

In phagocytes, the generation of reactive oxygen and nitrogen metabolites is crucial for microbial killing. These reactive molecules are originated in the products of the inducible enzyme systems NADPH oxidase and nitric oxide synthase, whose activity and/or expression are up-regulated after pathogen recognition by phagocytes and cytokine action. In the present studies, the capacity to generate superoxide was evaluated by determining the levels of the NADPH oxidase inducible and regulatory subunits gp91^{phox} and p47^{phox}. The compound AC201, initially studied, reduced the levels of these subunits, which would affect superoxide generation (Fig. 18, C). Instead, the naphthoquinone lapachol did not change the basal levels of these NADPH oxidase components and actually potentiated the levels of p47^{phox} already up-regulated by P₃CSK₄ treatment. Surprisingly, IFN- γ did not alter the expression of gp91^{phox} (Fig. 18, A), whose mRNA levels have been reported to be up-regulated by this cytokine in THP-1 cells (Newburger, Ezekowitz et al. 1988). However, the up-regulation may just be a short term effect, since the same report shows an important reduction in gp91^{phox} mRNA levels after 48 hours. This could be the case in the experiments here described, since effects were routinely determined after 4 days. In addition, a correlation between mRNA levels and protein expression is not necessarily true in many cases. Regarding NOS2 (iNOS), its levels were found increased after TLR2 agonism (Fig. 20, A). It was surprising that no increase in NOS2 expression by IFN- γ treatment was observed. IFN- γ is known to induce NO formation (Chan, Tanaka et al. 1995; Shtrichman and Samuel 2001), but this effect depends on the concentration and activity of the cytokine. Lapachol did not affect either baseline or TLR2 agonism-induced NOS2 levels (Fig. 20, A). IFN- γ -, TLR2 agonism- and *M. avium* infection-induced down-regulations of arginase-1 were observed (Fig. 20, A & B) that can be favouring NO formation by increasing arginine availability (Wu and Morris 1998). These potentially useful reductions were not affected by treatment with lapachol. The down-regulation of arginase-1 could in fact result in increased NO formation even in the absence of an

induction of NOS2. However, interpretation of some of the blottings is complicated by the absence of proper loading controls.

The fact that quinones can promote superoxide generation could result in cell damage and even toxicity. The extent of action of the naphthoquinone lapachol in this respect does not appear to be pronounced, since it did not increase the number of apoptotic cells. Besides, the absence of a lapachol-induced oxidative stress, indicated by unchanged MnSOD levels (Fig. 19), supports the idea of lapachol not being a particularly toxic quinone at the concentration used during the present studies.

Chapter 3 : IFN- γ responsiveness and antigen presentation

The immune response to mycobacterial infections involves different subsets of T cells. $\alpha\beta$ and $\gamma\delta$ T cells are two groups of T cells expressing different types of T cell receptor (TCR). $\alpha\beta$ T cell group is composed by three subsets, $CD4^+$, $CD8^+$ and CD1 restricted cells. During the attempts to control mycobacterial infection, $CD4^+$ T cells play a major role. After activation by mycobacterial antigens, T cells can be directly cytotoxic or can secrete the main macrophage activating cytokines IFN- γ and TNF- α , which enhance the intracellular killing of mycobacteria (Boom, Chervenak et al. 1992; Boom, Canaday et al. 2003; Felio, Nguyen et al. 2009). The first encounter with pathogens triggers a primary response where T cell proliferation is induced and T and B memory cells are generated. Memory cells are the basis of a quick response during a second contact with the same pathogen (Bonilla and Oettgen 2010).

T lymphocyte activation is dependent of two signals originated in the interaction of T cells with APCs during antigen presentation. The main signal is antigen specific, and is generated by TCR after binding to the antigen-MHC complex displayed on the surface of APCs. A secondary, but not less important, signal is antigen non-specific and is mediated by the interaction of the co-stimulatory molecules CD28, CTLA-4, and CD83-ligand on the T cell surface with CD80, CD83 and CD86 on the APC membrane. These events represent a link between innate and adaptive immunity, aimed at mounting an effective immune response against the invading organisms (Lenschow, Walunas et al. 1996; Greenwald, Latchman et al. 2002; Breloer and Fleischer 2008).

Protein antigens are presented by APCs in different ways depending of their origin. Bacteria are internalized by phagocytes and, as a consequence of a degradative process, their proteins are broken down to small peptides that associate with MHC II molecules. MHC II-peptide complexes translocate to the APC membrane where they are recognized

by CD4⁺ T cells. Mycobacterial components can also gain access to the cytosol of host cells and the antigens originated are presented on the APC surface associated with MHC I molecules, like any other intracellular antigen. The cross presentation phenomenon exerted by dendritic cells, represent another mechanism of mycobacteria antigens presentation associated with MHC I. Antigen-MHC I complexes are targeted by CD8⁺ cells. Mycobacterial lipids, e.g. mycolic acids, cannot associate with MHC I or II molecules due to their chemical characteristics. These molecules are presented associated with the hydrophobic groove of CD1 receptors. $\gamma\delta$ T cells recognise phospho-antigens which are presented by APCs in a still unknown manner (Moody, Besra et al. 1999; Boom, Canaday et al. 2003).

In brief, macrophages are efficient phagocytes, responsible for removing pathogens, erythrocytes and cell debris (Mosser and Edwards 2008). Moreover, they prime T cells for cell proliferation, synthesis of IFN- γ and immortalization (Pozzi, Maciaszek et al. 2005). IFN- γ secreted by activated lymphocytes enhances the antimicrobial and antigen presentation function of macrophages (Boehm, Klamp et al. 1997). Despite the macrophage ability to eliminate intracellular pathogens and present their antigens, pathogenic mycobacteria developed evading strategies that allow their proliferation within the host cell, as commented in the Introduction, section 9.2. (Chang, Linderman et al. 2005; Huynh and Grinstein 2007; Baena and Porcelli 2009).

The immunomodulatory effect of some natural products on antigen presentation has been studied. Smoke tobacco extracts (Gong and Chen 2003), the plant compound albacol (Liu, Shu et al. 2008), and cyanobacterial grassystatins (Kwan, Eksioglu et al. 2009) inhibit remarkably antigen presentation by APCs. The influence of lapachol on the expression of receptors/molecules involved in IFN- γ responses and antigen presentation by THP-1 macrophages was here studied.

1. Expression of IFN- γ R1

Two populations were detected that expressed surface IFN- γ receptor (IFN- γ R1) to different extents (Fig. 33, A). Treatment with lapachol or P₃CSK₄ increased the percentage of cells expressing high levels of IFN- γ R1 as shown by a shift from low to high IFN- γ R1-expressing cells (Fig. 33, B and C). Such up-regulation could be favouring responses to IFN- γ , considering the relative paucity of these receptors on the cell surface (Finbloom, Hoover et al. 1985). To determine whether the increase in the IFN- γ R1 surface signal was due to the translocation of pre-existing molecules from cytoplasmic storage vesicles or to new protein synthesis, the fluorescence signal of permeabilised cells (i.e. cytoplasmic + surface expression) was determined. Figure 34 shows histograms and bar graphs corresponding to control, lapachol-treated, TLR2-agonised and TLR2-agonised/lapachol-treated cells. No significant differences were observed among permeabilised cells that had been differently treated, as opposed to what was observed in intact cells (Fig. 33). This means that the lapachol- and TLR2 agonism-induced increases in IFN- γ R1 detected on the cell surface are originated in a decrease of IFN- γ R1 molecules stored intracellularly. The most likely explanation is a translocation of IFN- γ R1-containing cytoplasmic vesicles to the cell membrane, an event reported to occur in relationship with CD86 in macrophages (Smyth, Logan et al. 2005) and CR1 in neutrophils (Berger, Wetzler et al. 1991).

In view of the modulation of the surface expression of IFN- γ R1 by lapachol and TLR2 agonism, the surface expression of the following receptors known to be up-regulated by IFN- γ was examined: CD54 (ICAM-1), CD64 (Fc γ RI) and MHC II.

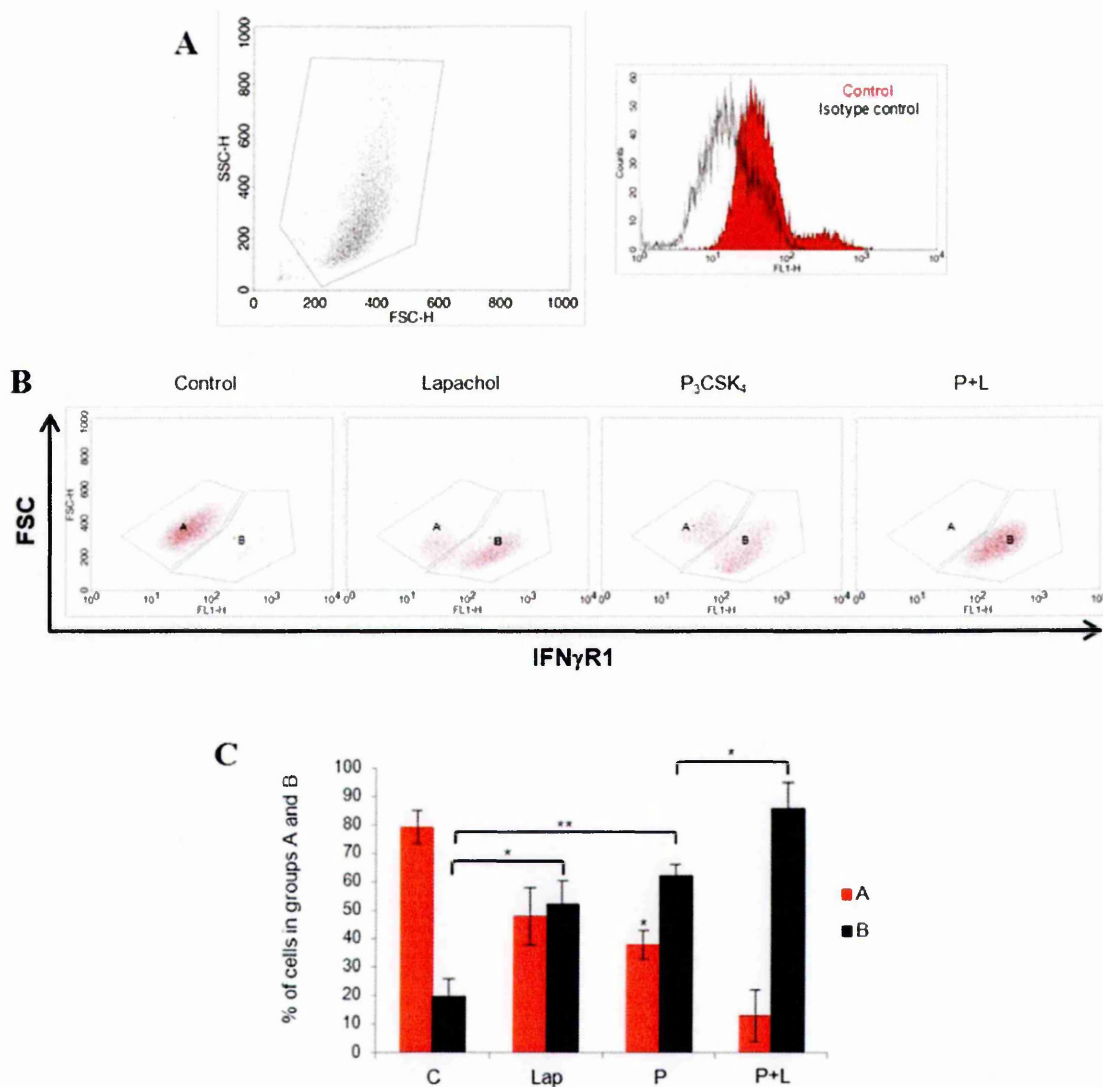


FIGURE 33. Effect of TLR2-agonism and lapachol treatment on the expression of surface IFN- γ R1 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of 300 nM P₃CSK₄ for 4 days and then for 4 additional days in the absence or presence of 32 μ g/mL lapachol. Cells were stained with FITC-conjugated mAb against IFN- γ R1 or the appropriate isotype controls, and analyzed by FACS. Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in C, and a representative histogram of a control (resting cells) sample and an isotype control.

B. Representative dot plots of forward scatter vs FITC fluorescence of two cell populations (A and B) expressing different levels of IFN γ R1.

C. Bar graphs showing the mean % of events corresponding to both the low (A) and high (B) expressing areas (mean \pm S.E., n=3, independent experiments). Statistical analysis was by ANOVA (* p \leq 0.05; ** p \leq 0.01).

C: Control; Lap: lapachol; P: P₃CSK₄; P + L: P₃CSK₄ + lapachol

2. Expression of CD64 (FcγRI)

The expression of FcγRI (CD64), involved in the high affinity binding and phagocytosis of IgG-opsonized pathogens, was not significantly affected by lapachol nor by TLR2 agonism, IFN-γ activation or *M. avium* infection (Fig. 24). The lack of response to IFN-γ could be due to the cytokine dose used or to the fact that the present experiments examine late phase events.

3. Expression of CD54 (ICAM-1)

CD54 is involved in cell attachment and migration. Its expression was not significantly affected by lapachol or any of the cell treatments (TLR2 agonism, IFN-γ) (Fig. 35, B). Similarly to CD64, the lack of response to IFN-γ could be due to the dose used or to the fact that the present experiments examine late phase events.

4. Expression of receptors involved in antigen presentation

4.1. Major histocompatibility molecule II (MHCII)

MHCII is involved in the presentation of exogenous antigens. Presentation is achieved by being recognized by T cell receptors on CD4⁺ T lymphocyte membranes. This molecule is expressed at very low levels on resting cells (Fig. 35, A). Incubation with lapachol alone and in association with IFN-γ or P₃CSK₄ induced a significantly higher expression of MHC II (Fig. 35, A) that could potentiate antigen presentation. The fact that IFN-γ did not result in an increase in MHCII was in line with the results obtained regarding CD64 and CD54, supporting the idea that it could be due to the IFN-γ dose used or to the fact that the present experiments examine late phase events.

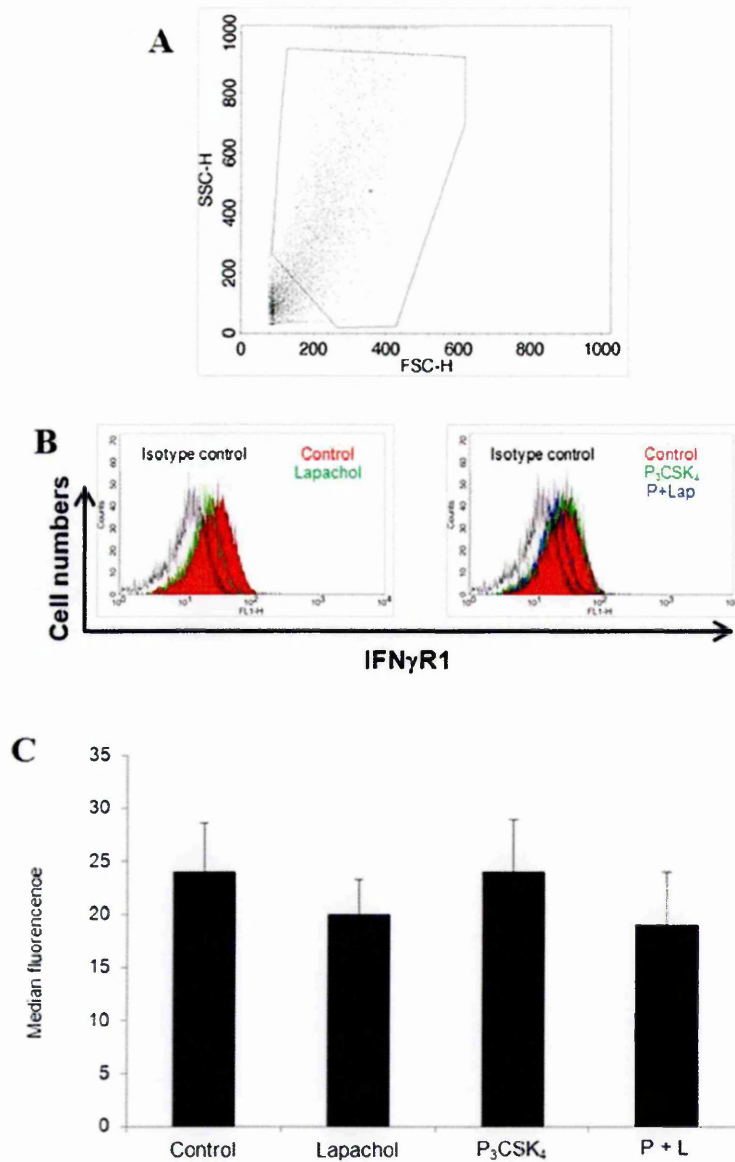


FIGURE 34. Effect of TLR2 agonism and lapachol treatment on the total (surface + cytoplasmic) expression of IFN- γ R1 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of 300 nM P₃CSK₄ for 4 days and then for 4 additional days in the absence or presence of 32 μ g/mL lapachol. Cells were then fixed and permeabilized with Fix & Perm® in order to detect both cytoplasmic and surface IFN- γ R1, and subsequently stained with FITC-conjugated mAb against IFN- γ R1. Appropriate isotype controls were run, and samples were analysed by FACS.

A. Dot plot of forward vs side scatter of permeabilized THP-1 macrophages, showing the cell gating region used for the analysis shown in **C**.

B. Representative histograms of IFN- γ R1 expression by permeabilized cells and of an isotype control.

C. Bar graph results are expressed as means \pm S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA. No significant ($p > 0.05$) differences were observed.

P+L or P+Lap: P₃CSK₄ + lapachol

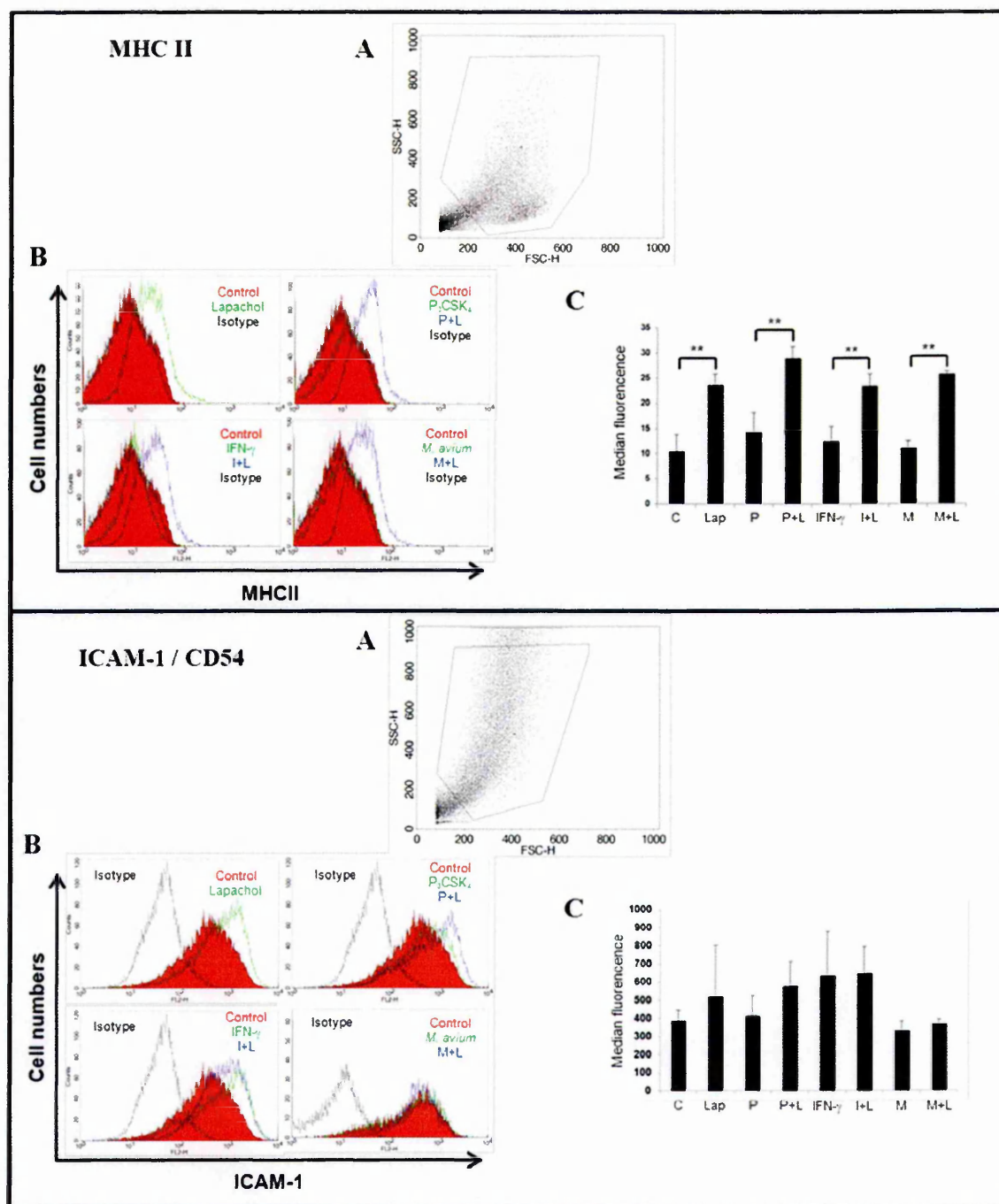


FIGURE 35. Effect of activation, *M. avium* infection and treatment with lapachol on the surface expression of MHCII and ICAM-1 (CD54) by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄, IFN- γ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 μ g/mL lapachol. Cells were stained with PE-conjugated mAb against MHCII (A) or ICAM-1 (B) or the appropriate isotype controls, and analyzed by flow cytometry (FACS). Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in C.

B. Representative histograms of isotype controls and of MHCII and ICAM-1 (CD54) expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means \pm S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA. ** $p \leq 0.01$.

C: Control; Lap: lapachol; P: P₃CSK₄; P + L: P₃CSK₄ + lapachol; I + L: IFN- γ + lapachol;

M: *M. avium*; M+L: *M. avium* + lapachol

4.2. Expression of co-stimulatory receptors

Macrophages are antigen presenting cells and as such they are engaged in the processing and presentation of pathogen antigens to T cells. CD83, CD80 and CD86 are members of a family of receptors that, together with MHC molecules, participate in the interaction with and activation of CD4⁺ or CD8⁺ T cells through their CD28 and CTLA-4 surface receptors. These so called co-stimulatory molecules trigger a signal on T cells that, together with MHC signaling, result in T cell activation and proliferation. Lapachol did not affect the expression of CD80 and CD83 but did inhibit the levels of the CD86 (Fig. 36, 37, 38), as did TLR2 agonism and *M. avium* infection.

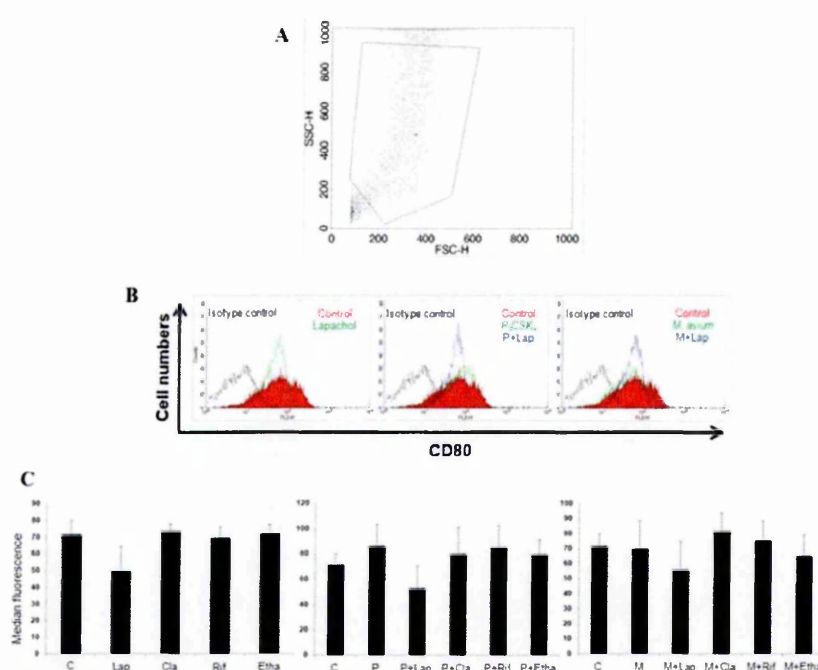


FIGURE 36. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface CD80 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against CD80 or the appropriate isotype controls, and analyzed by FACS. Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in **C**. **B.** Representative histograms of isotype controls and of CD80 expression in control, activated or infected or/and lapachol-treated cells, as indicated. **C.** Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA and showed no significant ($p > 0.05$) differences.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;
P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin; P+Rif: P₃CSK₄+Rifampicin; P+Etha: P₃CSK₄+Ethambutol;
M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;
M + Rif: *M. avium* + Rifampicin; M + Etha: *M. avium* + Ethambutol

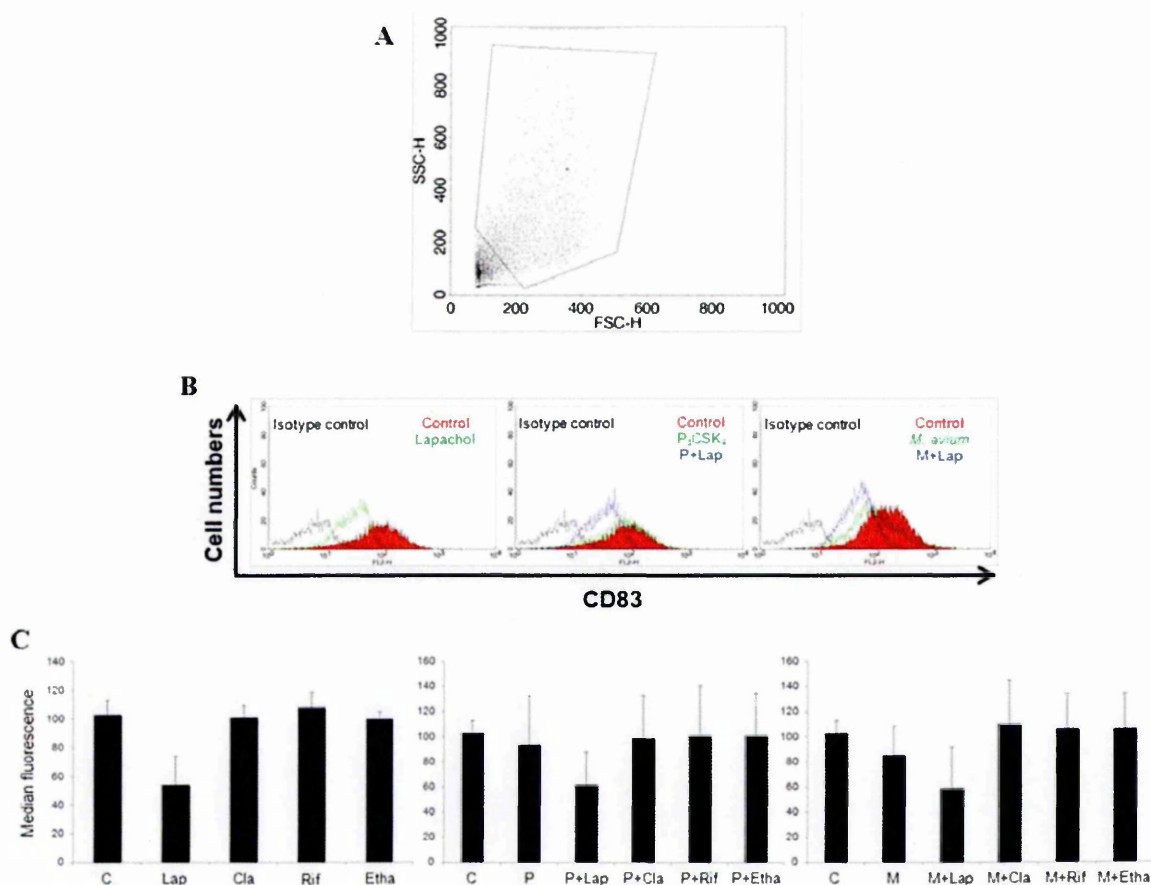


FIGURE 37. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface CD83 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against CD83 or the appropriate isotype controls, and analyzed by FACS. Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in C.

B. Representative histograms of isotype controls and of CD83 expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA and showed no significant ($p > 0.05$) differences.

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin; P+Rif: P₃CSK₄+Rifampicin; P+Etha: P₃CSK₄+ Ethambutol;

M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;

M + Rif: *M. avium* + Rifampicin; M + Etha: *M. avium* + Ethambutol

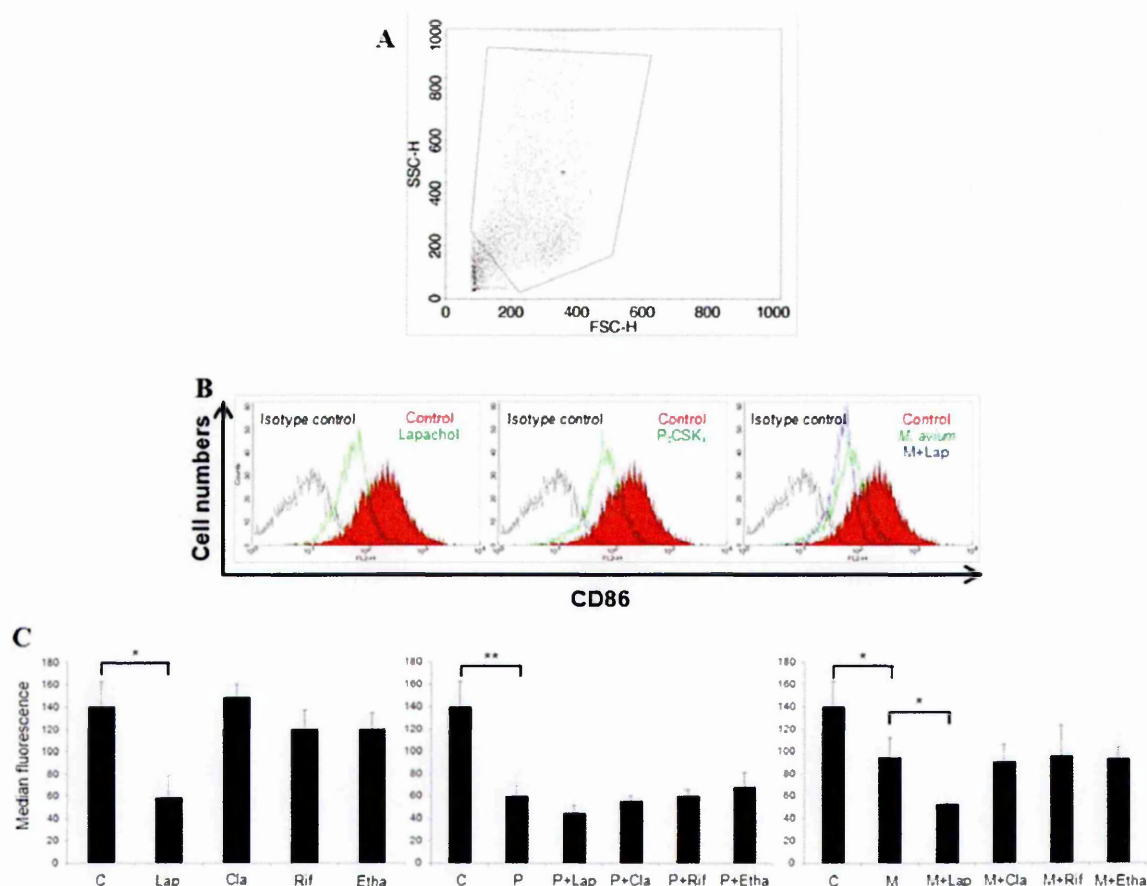


FIGURE 38. Effect of activation, *M. avium* infection and treatment with lapachol or traditional antibiotics on the expression of surface CD86 by THP-1 macrophages.

Macrophages were cultured in the absence (control) or presence of P₃CSK₄ or infected with *M. avium* for 4 days and then cultured for 4 additional days in the absence or presence of 32 µg/mL lapachol, 0.56 µg/mL clarithromycin, 1.6 µg/mL rifampicin or 0.35 µg/mL ethambutol. Cells were stained with PE-conjugated mAb against CD86 or the appropriate isotype controls, and analyzed by FACS. Number of total (ungated) events = 10 000.

A. Dot plot of forward vs side scatter of THP-1 macrophages showing the cell gating region used for the analysis shown in C.

B. Representative histograms of isotype controls and of CD86 expression in control, activated or infected or/and lapachol-treated cells, as indicated.

C. Bar graph results are expressed as means ± S.E. of the median fluorescence of 3 independent experiments. Statistical analysis was performed by ANOVA (* p ≤ 0.05 and ** p ≤ 0.01).

C: Control; Lap: Lapachol; Cla: Clarithromycin; Rif: Rifampicin; Etha: Ethambutol;

P: P₃CSK₄; P+Lap: P₃CSK₄+lapachol; P+Cla: P₃CSK₄+Clarithromycin; P+Rif: P₃CSK₄+Rifampicin; P+Etha: P₃CSK₄+Ethambutol;

M: *M. avium*; M + L: *M. avium* + Lapachol; M + Cla: *M. avium* + Clarithromycin;

M + Rif: *M. avium* + Rifampicin; M + Etha: *M. avium* + Ethambutol

IFN- γ is a pro-inflammatory cytokine involved in innate and acquired immune responses. Infections stimulate antigen-presenting cells to secrete IL-12, which attracts NK cells to the site of inflammation and induces them to secrete IFN- γ . IL-12 secretion by macrophages is enhanced by IFN- γ , and this increase in IL-12 further stimulates NK and T cells to secrete IFN- γ , in an amplifying mechanism. Macrophages are strongly activated by IFN- γ , which enhances pathogen recognition, antigen processing and presentation and microbicidal mechanisms (Schroder, Hertzog et al. 2004). The results obtained during this work regarding expression levels of key components of the NADPH oxidase and NO generation systems are shown in Chapter 2, sections 2 and 4. No up-regulation of gp91^{phox} and NOS2 expression by IFN- γ treated THP-1 macrophages was observed (Figs. 18 and 20). This could be a consequence of the fact that the experiments performed corresponded to responses at the 8th day of treatment with IFN- γ . In fact, Newburger et al (Newburger, Ezekowitz et al. 1988) found a reduction in mRNA levels for gp91^{phox} after 48 hours of treatment. The only effect of IFN- γ was an increase in the cytosolic levels of p47^{phox} (Fig. 18).

Concerning antigen presentation, a double signal induced by MHC molecules on the APC side and co-receptors on both APC and T cell sides are required. Lapachol did not affect the expression in macrophages of CD80 (Fig. 36) or CD83 (Fig. 37) and induced a decrease in CD86 (Fig. 38), as TLR2 agonism and *M. avium* infection did. *M. avium* infection has been previously reported to reduce CD86 levels (Mohaghehpour, Gammon et al. 1997; Rajavelu and Das 2008). Lapachol treatment, instead, led to a substantial increase in the cell surface expression of MHC II in resting, TLR2 agonised and *M. avium* infected macrophages (Fig. 35 A). Altogether, the results point to a possible improvement of antigen presentation by lapachol. Albacol, a plant-derived compound, and davallialactone, a compound isolated from mushrooms, produce a decrease in co-stimulatory molecules in DCs and RAW264.7 cells, respectively (Lee, Lee et al. 2008; Liu,

Shu et al. 2008) what inhibited T cell activation. The expression of CD54 (ICAM-1), shown by Zuckerman et al (Zuckerman, Pullen et al. 1998) to be able to induce proliferation of naïve T cells after binding to LFA-1 on the T cell surface, is not affected by lapachol in THP-1 macrophages (Fig. 35 B). This molecule could also participate in macrophage-T cell co-stimulation events.

Chapter 4. Proteomics approach to the effect of lapachol on macrophage activation.

IFN- γ or TLR activation of macrophages result in the stimulation of the production of inflammatory cytokines, enhance cell migration and tissue infiltration, phagocytosis and degranulation, endowing appropriately this cell to kill invading pathogens. All this is a consequence of transcription regulation events affecting gene expression (Ehrt, Schnappinger et al. 2001; Akira and Takeda 2004). Gene expression regulation results in changes in protein expression or modification. The primary goal of comparative proteomics is to detect and associate changes in protein expression to cellular changes, helping to elucidate the cause of the changes. Proteomic techniques are powerful tools to study protein expression and, according to the complexity of the analysis performed, can provide information about amounts, chemical modifications, cellular location and protein-protein interactions (Arthur 2003).

Proteomic approaches have been used for the study of changes in the protein profile of monocytes when they differentiate into macrophages, a crucial event in the innate response, or to describe the influence of stress or different inflammatory stimuli (Wang, Zhao et al. 2004). Differentiation of monocytes into macrophages is marked by changes in the profile of secreted and cellular proteins, as reported for U937 cells treated with PMA to become macrophages (Sintiprungrat, Singhto et al. 2010). Proteomic studies of IFN- γ -activated macrophage phagosomes shows delays in the acquisition of lysosomal hydrolases and peptidases for the gain of antigen presentation. This gain in antigen presentation depends on phagosomal cytoskeletal networks and on vesicle-trafficking proteins, Src kinases and calpain proteases (Trost, English et al. 2009).

Using proteomic techniques, alterations in protein expression in response to cell stress (Miura, Kano et al. 2005), TLR agonism (Xue, Yun et al. 2008) and challenges with

mycobacterial lipids (Shui, Gilmore et al. 2009) or with lipopolysaccharide (Gadgil, Pabst et al. 2003) have been reported. These techniques have also shown promising for the identification of possible anticancer drug targets (Hemers, Duval et al. 2005), as well as to elucidate the mechanism of cytotoxicity induced by anticancer drugs (Wang, He et al. 2005; Wang, He et al. 2006).

Among the different techniques used to separate, quantify and identify proteins, two-dimensional separation (isoelectrofocusing and polyacrylamide gel electrophoresis, 2D-PAGE) was described as early as 1969 and has considerably improved since then. It is a powerful technique for the resolution of complex protein mixtures, allowing quantification as well as identifications, including post-translational modifications. In association with mass spectrometry (single and/or tandem), protein masses and precise identifications are possible. The proteins present in the mixture are separated in the first dimension according to their isoelectric points, on a strip coated with a gel prepared as a fixed pH gradient (pI 3 to pI 11 or fractional ranges). The second dimension, dodecyl sulfate (SDS) gel electrophoresis, separates according to their molecular weight the proteins that had been first resolved on the pH gradient strips (Ong and Pandey 2001; Issaq and Veenstra 2008). This is done by placing each strip horizontally over the electrophoresis gel. The final result is a two-dimensional array of proteins spots (a protein 2D map) that can be visualized by staining the gel. Sensitive staining techniques (e.g. colloidal Coomassie, fluorescent dyes) allow the detection of protein spots containing down to 1ng (Candiano, Bruschi et al. 2004). The number of protein spots that can be detected is limited by the maximum of protein that can be loaded on the pH gradient strip. This limitation can be partially bypassed by performing cell fractionation before the bi-dimensional separations. In this way, the maximum protein load corresponds to a cell fraction. Since cell fractions contain fewer proteins than the whole cell, the cell fractionation procedure increases the total number of protein spots that can be detected, i.e. the number of spots in the different cell fractions added together. Understandably, the total number of protein spots detected is

smaller than the total number of proteins in a cell. The human genome sequence shows 20000 to 25000 protein-coding genes (Consortium 2004), from which at least 10000 proteins are estimated to be expressed in mammalian cells (Gorg, Weiss et al. 2004). This number of proteins will result in a higher number of spots due to protein modifications, isoforms and degradation fragments. Approximately 3000 protein spots can be detected by bi-dimensional (IEF/SDS-PAGE) separation if the cell is previously separated in cytosolic (soluble proteins) and membrane-associated (membrane-bound cell structures = plasma membrane + cytoplasmic organelles) fractions, as was the case in the present work. The number of protein spots detected, therefore, is $\leq 15\%$ of the total, which means that many proteins of low abundance are not being detected. Nevertheless, the number of protein molecules whose expression changes can be examined in relationship with cell stimulation or drug treatments is much greater than that provided by any other method. Techniques like e.g. FACS or western blotting combined with a high sensitivity method of visualization can detect proteins of low abundance but only with prior knowledge of their existence and subjected to the availability of antibodies in the market. Antibody arrays cover a limited number of proteins within specific fields. Transcriptomics does provide wide ranging information, with chips allowing the screening of the expression of about 29000 genes (Perco, Muhlberger et al. 2010). Nevertheless, changes at the RNA level do not always correlate with protein expression. Besides, information about protein isoforms, post-translational modifications or proteolysis is not obtained.

Bi-dimensional separations can also be used for the detection of proteins of lower abundance with the addition of a protein mining step where abundant proteins are excluded from a cell extract, yielding an extract enriched in the less represented ones (Boschetti and Righetti 2008). A cell sample should therefore be divided for analysis: a smaller portion for the detection of abundant proteins and a larger portion for the selection of low abundant proteins. Both portions are separately processed using the bi-dimensional techniques.

Macrophage functions and activation will in most cases be reflected by changes in protein patterns and expression. The proteomic section of the present work studied the influence of the naphthoquinone lapachol on the bi-dimensional patterns of protein expression corresponding to: 1) resting cells, 2) TLR2-agonised cells, and 3) IFN- γ -treated cells. Resting cells or cells that had been treated with the lipopeptide P₃CSK₄ (TLR2 agonist) or IFN- γ were treated or not with lapachol, harvested and separated into cytosolic and membrane fractions by high speed centrifugation. Proteins in each of the fractions were separated by isoelectrofocusing (IEF) followed by SDS-PAGE, obtaining 2D-gels. Duplicate gels were stained and images were taken by scanning. The images were analysed by means of a commercial program (Ludesi AB, Lund, Sweden: www.ludesi.com) that aligns and normalizes all gels and quantifies all the protein spots. Each spot is given a number that is the same in all gels and corresponds to a unique position. The images corresponding to each cell situation (resting, TLR2-agonised or IFN- γ -treated, in the absence or presence of lapachol, i.e. a total of 6 situations) and each cell fraction (cytosolic or membrane, i.e. making a total of 12 groups of 2 gel images each) can be compared following the researcher's criteria regarding group comparisons to be made. The operator also establishes parameters such as statistical significance ($p < 0.05$ in the studies here reported) and the extent of the differences between spots in different groups (≥ 1.5 -fold in the present studies). A list of spot locations and intensity differences is obtained (Ludesi Report), along with the corresponding bar or spread plot graphs showing the differences. On that basis it is decided which protein spots to excise from gels and identify by MS after tryptic proteolysis.

1. Changes induced by lapachol in resting cells

The number of protein spots whose expression level was altered by lapachol by ≥ 1.5 -fold in resting macrophages was determined. Changes were found in 25/1450 cytosolic spots (1.7%) and 60/1293 membrane spots (4.6%) (Figure 39). Protein identification was

not performed because it was considered to be more relevant to examine the effect of lapachol in relationship with macrophage activated states.

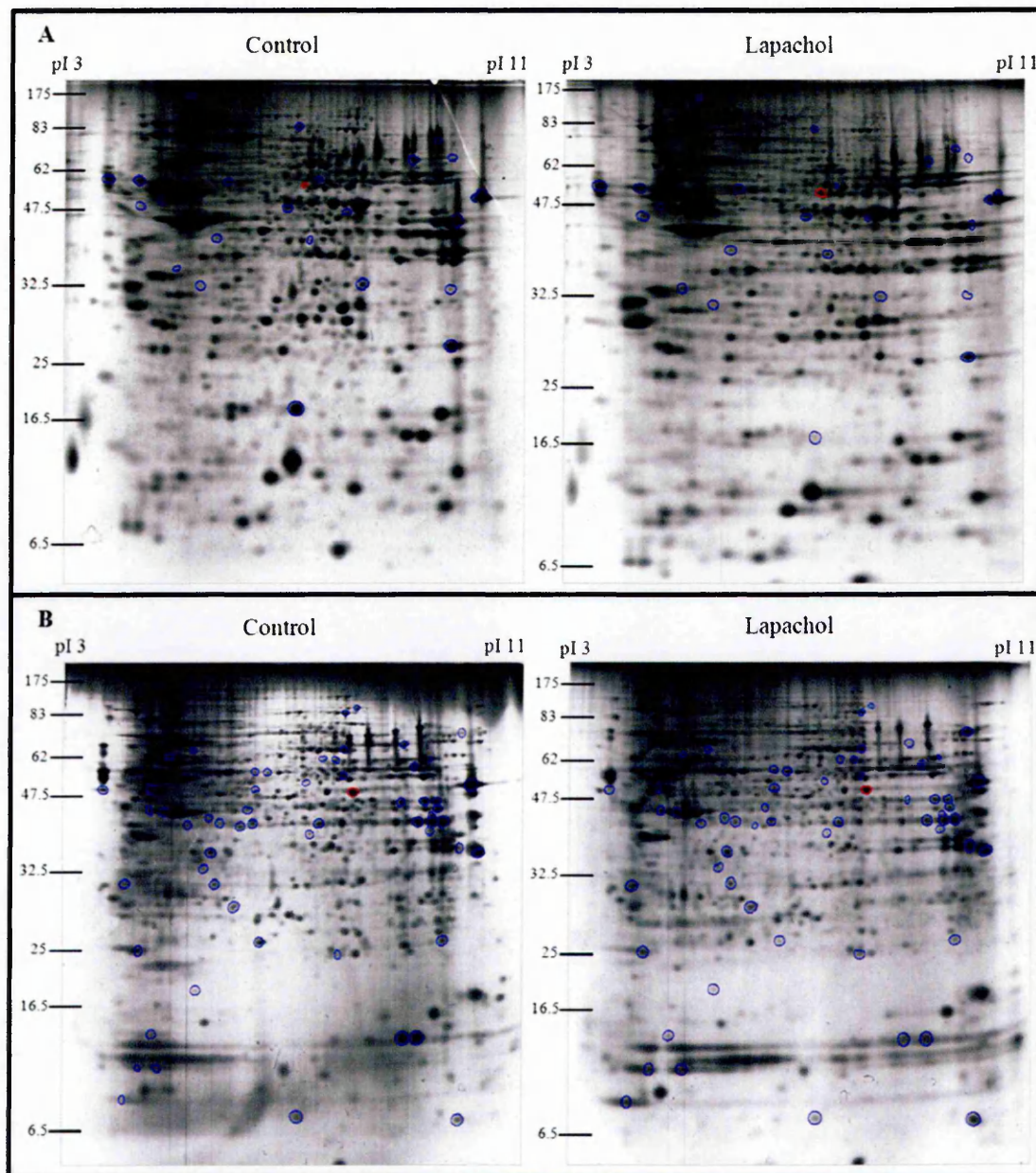


FIGURE 39. Representative cytosolic (A) and membrane (B) protein maps (2D-PAGE) of THP-1 macrophages untreated (Control) or treated with lapachol.

Cells were cultured in the absence of additions for 8 days (control); or without additions for 4 days and then with lapachol (32 μ g/mL) for 4 additional days (lapachol).

2D-PAGE: 1st dimension IEF, 2nd dimension SDS-PAGE (mol. masses of markers in kDa). Staining: colloidal Coomassie. The corresponding images were analysed, yielding 1293-1450 spots per gel.

The spots encircled blue or red correspond to lapachol-regulated proteins and were not identified. The red circles are the result of a technical feature of the gel image analysis program (Ludesi Redfin); they have the same meaning as the blue ones.

2. Influence of lapachol on IFN- γ -regulated protein expression levels

IFN- γ modulated the intensity of 111 out of the 1352 cytosolic protein spots detected on gels by ≥ 1.5 -fold, comparing IFN- γ -treated with untreated cells. Treatment of IFN- γ -activated cells with lapachol resulted in ≥ 1.5 -fold changes, either increases or decreases, in the intensity of 8 out of the 111 cytosolic protein spots regulated by IFN- γ (Figs. 40 and 42). Regarding membrane protein spots, 65 out of 1248 were found to be regulated by IFN- γ by ≥ 1.5 -fold, comparing IFN- γ -treated with untreated cells. Only 6 out of the 65 were further affected by lapachol by ≥ 1.5 -fold (Fig. 41 and 43).

Out of the total of 14 protein spots (8 in cytosolic and 6 in membrane fractions) of IFN- γ -regulated proteins affected by treatment with lapachol, 10 proteins could be identified by MS: 7 from the cytosol and 3 from the membrane fraction (Table 3). The changes in intensity of the corresponding spots are illustrated in Fig. 42 (cytosolic spots) and Fig. 43 (membrane spots), which show both the section of the gels where each spot is present and their quantification bar graphs. The extent of the changes observed varied considerably among spots, with the meaning of infinity ratios being the total absence of a spot in one of the situations being compared with each other. The characteristics of the proteins identified are summarized in Table 3.

The 10 IFN- γ -regulated proteins affected by lapachol identified were classified in terms of cellular function (Table 3), based on information from the protein knowledge database Swiss-Prot/TrEMBL (<http://www.expasy.ch>). As shown in Figure 44, the largest group of proteins was that of chaperones (40%), involved in protein folding. The other proteins were distributed among proteolysis, glycolysis and actin-binding.

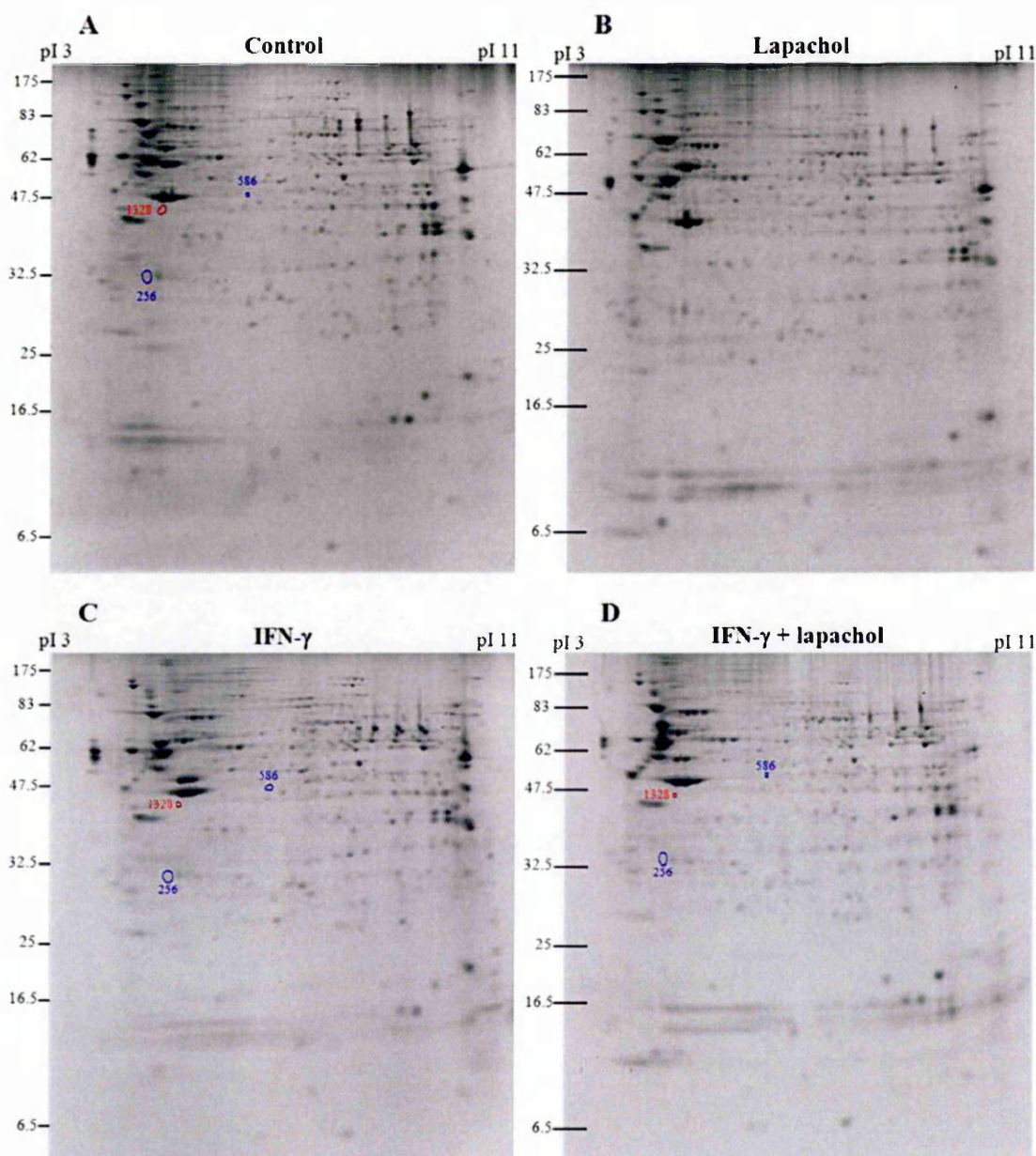


FIGURE 41. Representative membrane protein maps (2D-PAGE) of THP-1 macrophages untreated (A) or treated with lapachol (B), IFN- γ (C) or IFN- γ + lapachol (D).

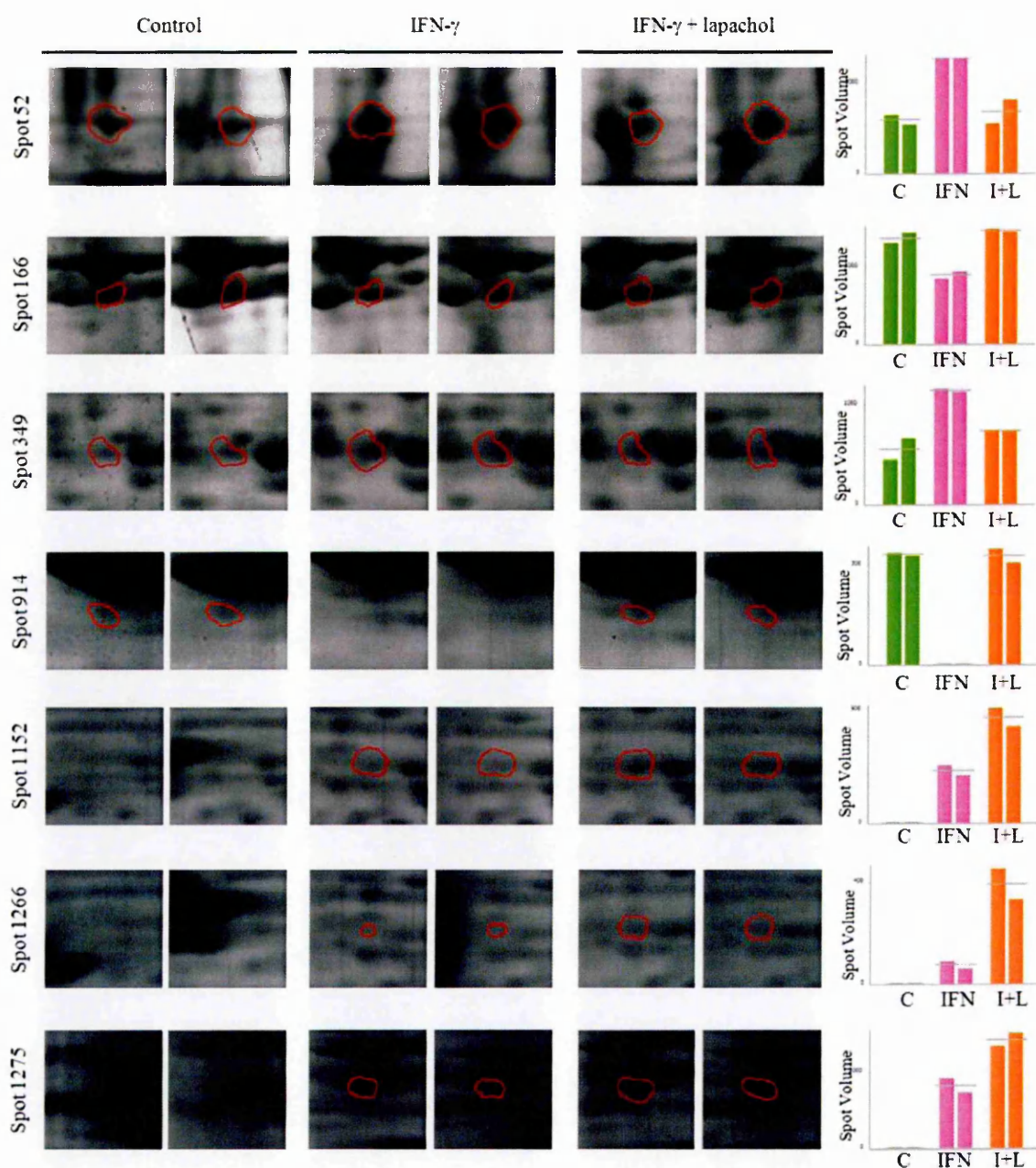
Cells were cultured in the absence of additions for 8 days (A), without additions for 4 days and then with lapachol (32 μ g/mL) for 4 days (B), in the presence of IFN- γ for eight days (C); in the presence of IFN- γ for 8 days and of lapachol (32 μ g/mL) from day 4 to 8 (D).

2D-PAGE: 1st dimension IEF, 2nd dimension SDS-PAGE (mol. masses of markers in kDa). Staining: colloidal Coomassie. The corresponding images were analysed, yielding 1250-1350 spots per gel.

The spots encircled blue or red correspond to IFN- γ -regulated proteins that were affected by lapachol, and were identified (Table 3). The red circles are the result of a technical feature of the image analysis program (Ludesi Redfin); they have the same meaning as the blue ones.

Cellular fraction	Spot Nr	Protein	Swiss Prot Acc. Nr	Cellular function	Score	Exp MW/pI	Theor MW/pI	Number of peptides detected	Fold change	
									IFN- γ vs. Control	I+L vs. IFN- γ
Cytosol	52	Pyruvate kinase	P14618	Glycolysis	582	72.4/8.0	57.8/7.95	18	\uparrow 2.15	\downarrow 1.87
Cytosol	166	Adenyl cyclase-associated protein 1-CAP1	Q01518	Cytoskeleton	674	66.0/8.5	51.7/8.29	18	\downarrow 1.53	\uparrow 1.64
Cytosol	349	Alpha enolase	P06733	Glycolysis	745	54.9/5.7	47.0/6.99	17	\uparrow 2.06	\downarrow 1.55
Cytosol	914	Scavrin-specific protease 6 (SUMO-1)	Q9GZR1	Ubiquitination	133	43.6/4.9	51.5/5.78	9	\downarrow ∞	\uparrow ∞
Cytosol	1152	Calreticulin	P27797	ER stress	112	63.0/5.2	46.4/4.29	7	\uparrow ∞	\uparrow 2.00
Cytosol	1266	Protein disulfide-isomerase A3 (PDI)	P30101	ER stress	114	63.0/5.1	54.2/5.61	17	\uparrow ∞	\uparrow 5.28
Cytosol	1275	Heat shock protein 90-beta (HSP90)	P08238	ER stress	507	117/5.4	83.1/4.97	19	\uparrow ∞	\uparrow 1.73
Membrane	256	Cathepsin B	P07858	Proteolysis	144	31.6/4.9	27.8/5.23	6	\downarrow 2.41	\uparrow 2.38
Membrane	586	DnaJ homolog subfamily B, #11	Q9UBS4	ER stress	168	48.9/6.0	38.1/5.81	13	\uparrow ∞	\downarrow ∞
Membrane	1328	Beta-actin-like protein 2	Q562R1	Cytoskeleton	92	45.7/5.0	42/5.39	10	\downarrow 7.31	\downarrow ∞

Table 3. Proteins differentially expressed upon IFN- γ -treatment whose expression levels were affected by lapachol. Differentially expressed proteins due to IFN- γ treatment for 8 days (IFN- γ vs. Control column, values \geq 1.5-fold), the expression of which changed when lapachol was present during the last 4 days (I+L vs. IFN- γ column, values \geq 1.5-fold). Protein names and functions are according to UniProt Knowledge base (SwissProt + TrEMBL) (<http://expasy.org/sprot/>). I+L : IFN- γ + lapachol; Exp. : Experimental; Theor. : Theoretical; MW : Molecular weight; pI: Isoelectric point.



spot 52 : Pyruvate kinase;
spot 914 : SUMO-1;
spot 1275 HSP90

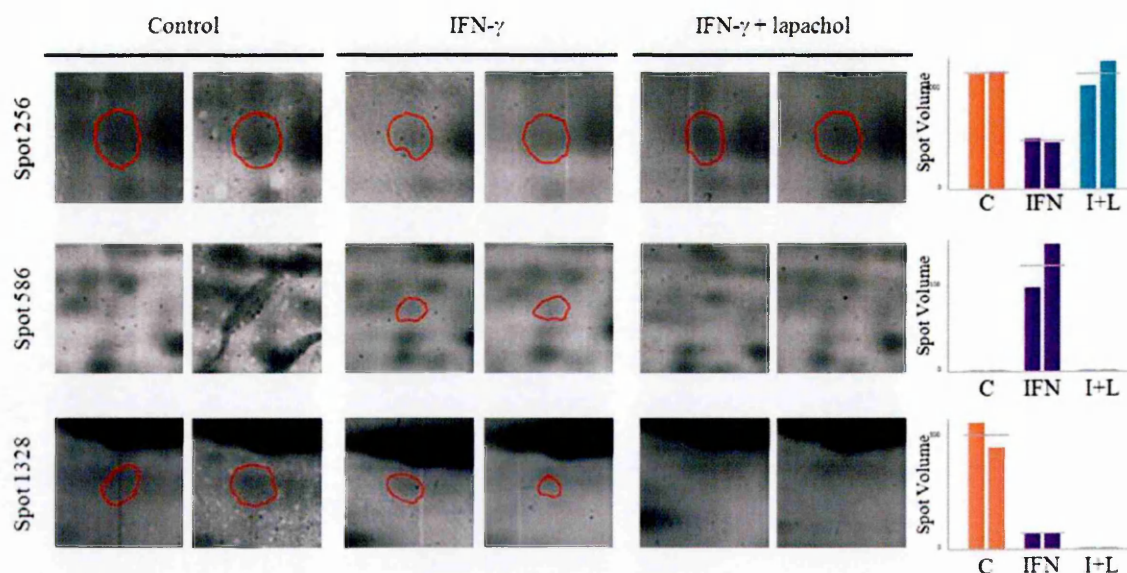
spot 166 : CAP-1;
spot 1152 : Calreticulin;

spot 349: Alpha-enolase;
spot 1266 : PDI;

FIGURE 42. Detail of the alterations induced by lapachol on IFN- γ -regulated cytosolic protein expression.

Cells were cultured in the absence of additions for 8 days (**Control**), in the presence of IFN- γ for eight days (**IFN- γ**); with IFN- γ for 8 days and additionally lapachol (32 μ g/mL) from day 4 to 8 (**IFN- γ + lapachol**). Cytosolic proteins were analysed by IEF/ SDS-PAGE, in duplicate. Gel images were subjected to quantitative analysis (www.ludesi.com), which includes a normalization step. The figure illustrates the areas of the gels showing proteins (red encircled spots) regulated by IFN- γ activation, the expression of which was affected by lapachol. The bar graphs show the quantification of the spots, in each case.

C: Controls (untreated cells); IFN: IFN- γ ; I+L: IFN- γ + lapachol.



spot 256 : Cathepsin B; **spot 586** : DnaJ homolog subfamily B;
spot 1328: β -actin-like protein 2.

FIGURE 43. Detail of the alterations induced by lapachol on IFN- γ -regulated membrane protein expression.

Cells were cultured in the absence of additions for 8 days (**Control**), in the presence of IFN- γ for eight days (**IFN- γ**); with IFN- γ for 8 days and additionally lapachol (32 μ g/mL) from day 4 to 8 (**IFN- γ + lapachol**). Membrane proteins were analysed by IEF/ SDS-PAGE, in duplicate. Gel images were subjected to quantitative analysis (www.ludesi.com), which involves a normalization step. The figure illustrates the areas of the gels showing proteins (red encircled spots) regulated by IFN- γ activation, the expression of which was affected by lapachol. The bar graphs show the quantification of the spots, in each case.

C: Controls (untreated cells); IFN: IFN- γ ; I+L: IFN- γ + lapachol.

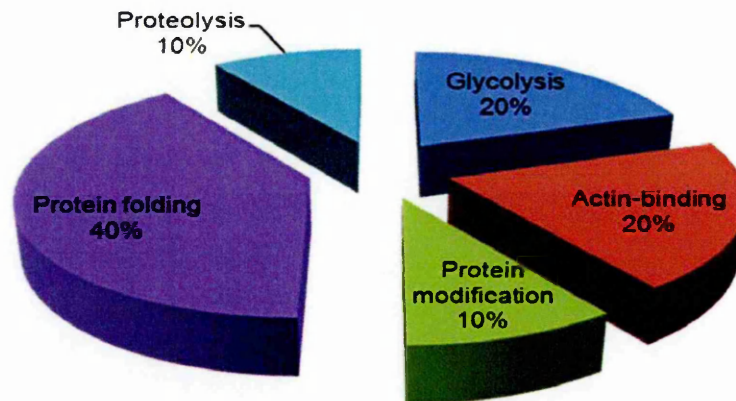


FIGURE 44. Functional classification of the IFN- γ -regulated proteins the expression of which was influenced by lapachol.

Pie charts representing the distribution of identified proteins according to their biological function. Assignments were made based on information from the Swiss-Prot/TrEMBL protein database (<http://www.expasy.ch>).

2.1. Protein folding

Four proteins classically involved in ER protein folding were identified (40%): protein disulfide-isomerase A3 (PDI), calreticulin, heat shock protein 90 (HSP90) and DnaJ homolog subfamily B #1. Three of these proteins, namely PDI (spot 1266), calreticulin (spot 1152) and HSP90 (spot 1275), were found in the cytosolic fraction of IFN- γ -treated but not in non-treated (control) cells, and increased further in the presence of lapachol (Table 3, Fig. 42). The unusual cytosolic localization of these proteins is not likely to be the result of ER damage during cell disruption because a very abundant ER protein, grp78, was not found up-regulated. PDI A3, also known as ERp57, is thought to be involved in Stat3 complexing and in disulfide bond cleavages coupled to protein degradation in the cytosol of Hep3B cells (Turano, Coppari et al. 2002). It has also been reported to be present in the cytosol of breast cancer cells, where is cleaved by caspase-3 and -7 during induced apoptosis (Na, Park et al. 2007). The lapachol-induced up-regulation of cytosolic

PDI A3, which was detected uncleaved and at the correct pI position in the 2D protein map (Table 3), is in agreement with the lack of an apoptotic effect of lapachol.

Calreticulin is known to express non-ER functions (Gold, Eggleton et al. 2010) such as to retrotranslocate from the ER to the cell cytosol (Afshar, Black et al. 2005), where it can be arginylated and associate to stress granules (Carpio, Lopez Sambrooks et al. 2010). The results here obtained indicate that the cytosolic calreticulin up-regulated by lapachol (Fig. 42) has a pI=5.2, against a theoretical value of 4.3 (Table 3), and this is compatible with a protein modification conferring positive charges such as arginylation (Karakozova, Kozak et al. 2006).

HSP90 is a heat-shock protein known to associate with MHCI and II molecules during cargo loading. HSP90 inhibition studies (Rajagopal, Bal et al. 2006; Callahan, Garg et al. 2008) have shown that inhibited macrophages fail to load antigen on MHCI and II, which could compromise CD4⁺ or CD8⁺ activation. The enhancement of the expression of HSP90 by IFN- γ and the further increase induced by lapachol (Fig. 42) could therefore be beneficial.

An up-regulation of DnaJ homolog (spot 586) was detected in the membrane fraction, which is compatible with its ER localization. In this case, its IFN- γ -induced up-regulation was suppressed by lapachol treatment (Fig. 43). The luminal domain of this protein stimulates the basal ATPase activity of grp78/BiP, which may favour grp78 monomerization (Chevalier, Rhee et al. 2000). Lapachol could therefore be interfering to some extent with grp78 function.

2.2. Glycolysis

IFN- γ increased the expression of two cytosolic proteins involved in glycolysis: α -enolase (spot 349) and pyruvate kinase (PK, spot 52), and the increase reverted to control values by treatment with lapachol (Table 3). The change observed for enolase only

concerned an acidic form of pI 5.7, compared to pI 7.0 for the unmodified form, and not the rest of enolase isoforms. It is therefore difficult to predict the possible consequences of the isoform down-regulation observed. The effect of lapachol could be related to an interference of a post-translational modification of enolase. The lapachol-induced reduction in PK levels corresponded to the molecular form of normal pI (Kang, Kim et al. 2006). It could have some effect on glycolysis and the subsequent ATP generation, since PK is a regulatory enzyme (Munoz and Ponce 2003; Mazurek, Boschek et al. 2005). The present result is the first report about a positive regulation of PK expression by IFN- γ . Since the generation of an excess of pyruvate has been reported to participate in an anti-oxidant action (Miwa, Fujii et al. 2000), the IFN- γ -induced increase in PK could have a protective effect against possible oxidative damages elicited by IFN- γ . The lapachol-induced return of PK levels to those of untreated cells could therefore have some secondary effect on the protection against free radicals. The main protective mechanisms, due to MnSOD and CuZnSOD, are not affected by lapachol (Chapter 2, section 3; Fig. 19).

2.3. Post-translational modifications

A protein involved in post-translational modification, sentrin-specific protease 6 or SUMO-1 (spot 914), was identified. Its cytosolic expression was down-regulated to non-detectable levels by IFN- γ , and restored to levels similar to control after lapachol treatment (Table 3). SUMO family proteins modify many proteins involved in cellular functions such as transcription, genome integrity, nuclear transport and signal transduction by forming conjugates (Seeler and Dejean 2001; Johnson 2004). Although most SUMO conjugates are nuclear proteins, some are cytoplasmic like SUMO-RanGAP1 and SUMO-I κ B α . SUMO-RanGap1 is the most abundant SUMO conjugate in vertebrate cells. It is a GTPase activating protein acting on Ran. Ran plays a central role in nucleocytoplasmic transport, being found on the cytoplasmic fibrils of nuclear pore complexes, and participates in mitosis events. However, it is not yet clear what role sumoylation of RanGAP1 could play

in nuclear transport. Therefore, it is difficult to speculate on the meaning of the IFN- γ -induced down-regulation of SUMO-1 observed and the restoration of its levels by lapachol treatment.

NF- κ B activity is dependent on the ubiquitin-dependent degradation of the NF- κ B inhibitor I κ B. SUMO prevents I κ B α degradation by conjugation (sumoylation), leading to a reduction in NF- κ B-dependent transcriptional activity (Desterro, Rodriguez et al. 1998). It can be speculated that by reducing SUMO-1 availability, IFN- γ would promote NF- κ B transcriptional activity, which is associated to the signalling of many pathways including TLR. By restoring SUMO-1 levels to normal, lapachol could somehow hamper NF- κ B transcriptional activity. However, lapachol did not affect the TLR2 agonism-induced secretion of TNF- α , a typical NF- κ B-mediated process (Collart, Baeuerle et al. 1990).

2.4. Proteolysis

Cathepsin B (CatB, spot 256) is a lysosomal cysteine protease that degrades exogenous and cellular proteins and can also work as an endopeptidase (Mort and Buttle 1997). CatB can also be found in the cell cytosol, plasma membrane and pericellular spaces where it plays a role in tumour migration and invasiveness (Wickramasinghe, Nagaraj et al. 2005). The proteolytic activity of CatB is not required for antigen presentation by MHCII molecules, since cathepsin B deficient mice were able to present foreign antigens (Deussing, Roth et al. 1998). The results presented here show an IFN- γ -induced reduction in CatB and differ from those obtained in THP-1 cells but using a 5-fold higher IFN- γ concentration and measuring mRNA expression (Li and Bever 1997). The association of IFN- γ with lapachol resulted in an increase in CatB levels back to those in untreated cells. CatB has been recently reported to be involved in the cytoplasmic traffic of TNF- α -containing vesicles to the plasma membrane (Ha, Martins et al. 2008). Thus, lapachol could facilitate the secretion of this cytokine.

2.5. Actin-binding

The expression of adenyl cyclase-associated protein-1 (CAP-1, spot 166) was down-regulated by IFN- γ (Table 3) and lapachol restored its levels to those of untreated cells. This protein participates in the mechanism of cell polarization, which requires a cytoskeletal rearrangement mediated by CAP1 thanks to its capacity to regulate the F-/G-actin ratio. Cells deficient in CAP are bigger, in keeping with abnormalities in cytoskeleton structure (Hubberstey and Mottillo 2002). The reduction in CAP1 expression by IFN- γ and its reversion by lapachol could result in transient, opposite effects on cell migration, although the extent of the changes observed (Table 3 and Fig. 42) is modest and such effects might not show. Indeed, lapachol did not cause relevant changes in cell shape or size of the THP-1 macrophages here used, as illustrated in Fig. 12.

β -actin-like protein 2 (spot 1328) was strongly down-regulated (7-fold) by IFN- γ in membrane fractions, and still further by lapachol treatment. This protein spot is located below the bulk of actin in 2D-gels, and is comparatively very scarce (Fig. 44). Because of its smaller molecular mass, it could be a product of partial degradation from actin. Since total actin levels were not affected by IFN- γ activation (Fig. 17, C), and cell shape and size were not seen to be affected either, it seems unlikely that β -actin-like protein 2 be of any relevance regarding the cytoskeleton unless some yet non described regulatory function is discovered for it.

3. Influence of lapachol on P₃CSK₄-regulated protein expression levels

TLR2 agonism with the lipopeptide P₃CSK₄ induced ≥ 1.5 -fold changes in the intensity of 150 cytosolic protein spots out of a total of 1400. When TLR2-agonised cells were treated with lapachol, the intensity of 22 of those 150 spots changed further by ≥ 1.5 -fold, either increasing or decreasing (Fig. 45). The intensity of 115 protein spots from the membrane fraction, out of a total of 1265, was changed by P₃CSK₄ treatment by ≥ 1.5 -fold, either in a positive or negative way. Treatment with lapachol resulted in the modification of the levels of 18 out of those 115 membrane protein spots (Fig. 46).

The proteins corresponding to 11 out of the total of 40 spots that were affected by lapachol (22 cytosolic and 18 membrane-associated) could be identified by MS. The changes in intensity of the corresponding spots are illustrated in Fig. 47 (cytosolic spots) and Fig. 48 (membrane spots). Both the section of the gels where each spot is present and their quantification bar graphs are illustrated. The extent of the changes observed was very variable, infinity ratios corresponding to the total absence of a spot in one of the situations being compared with each other. The characteristics of the proteins identified are summarized in Table 4.

The proteins identified were classified in terms of cellular function based on information from Swiss-Prot/TrEMBL protein database (<http://www.expasy.ch>). As shown in Fig. 49, they are distributed in the following cellular functions: actin-binding (28%), metabolism (27%), proteolysis (27%), ER stress (9%) and oxido-reduction (9%).

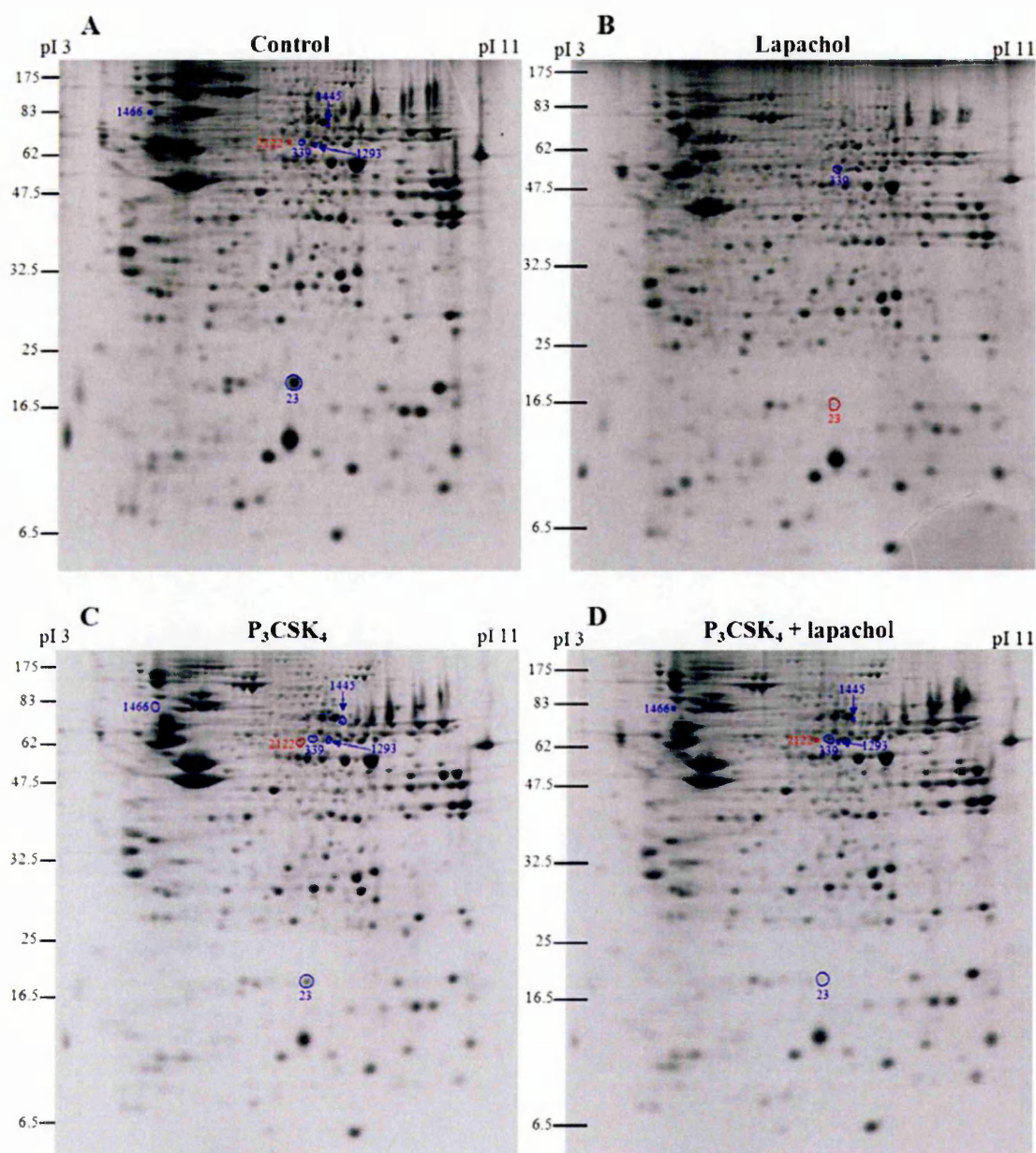


FIGURE 45. Representative cytosolic protein maps (2D-PAGE) of THP-1 macrophages untreated (A) or treated with lapachol (B), P₃CSK₄ (C) or P₃CSK₄ + lapachol (D).

Cells were cultured in the absence of additions for 8 days (A); without additions for 4 days and then with lapachol (32µg/mL) for 4 days (B); in the presence of P₃CSK₄ (300 nM) for eight days (C); with P₃CSK₄(300 nM) for 8 days and lapachol (32µg/mL) from day 4 to 8 (D).

2D-PAGE: 1st dimension IEF, 2nd dimension SDS-PAGE (mol. masses of markers in kDa). Staining: colloidal Coomassie. The corresponding images were analysed, yielding 1400-1450 spots per gel.

The spots encircled blue or red correspond to P₃CSK₄-regulated proteins that were affected by lapachol, and were identified (Table 3). The red circles are the result of a technical feature of the gel image analysis program (Ludesi Redfin); they have the same meaning as the blue ones.

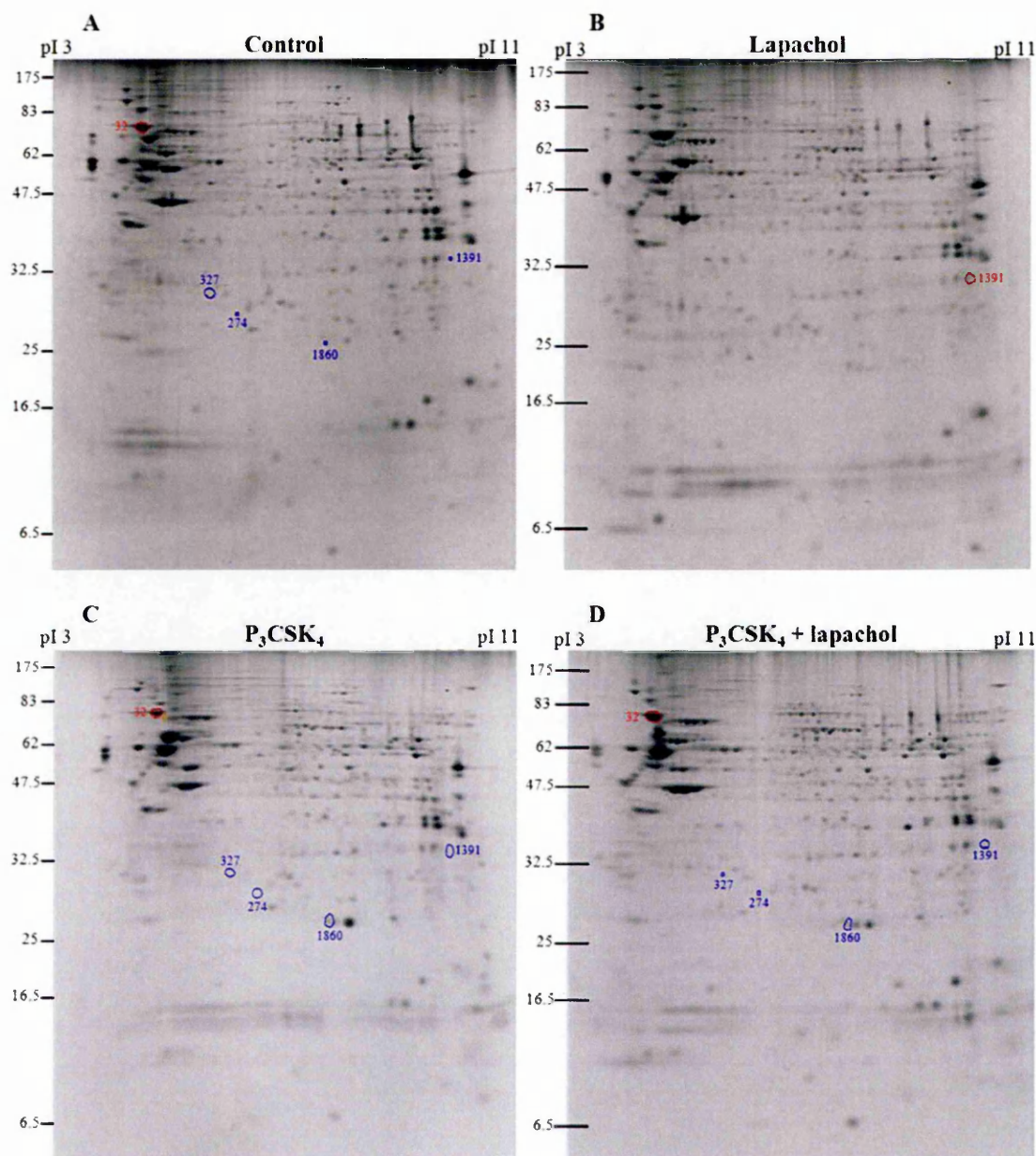


FIGURE 46. Representative membrane protein maps (2D-PAGE) of THP-1 macrophages untreated (A) or treated with lapachol (B), P₃CSK₄ (C) or P₃CSK₄ + lapachol (D).

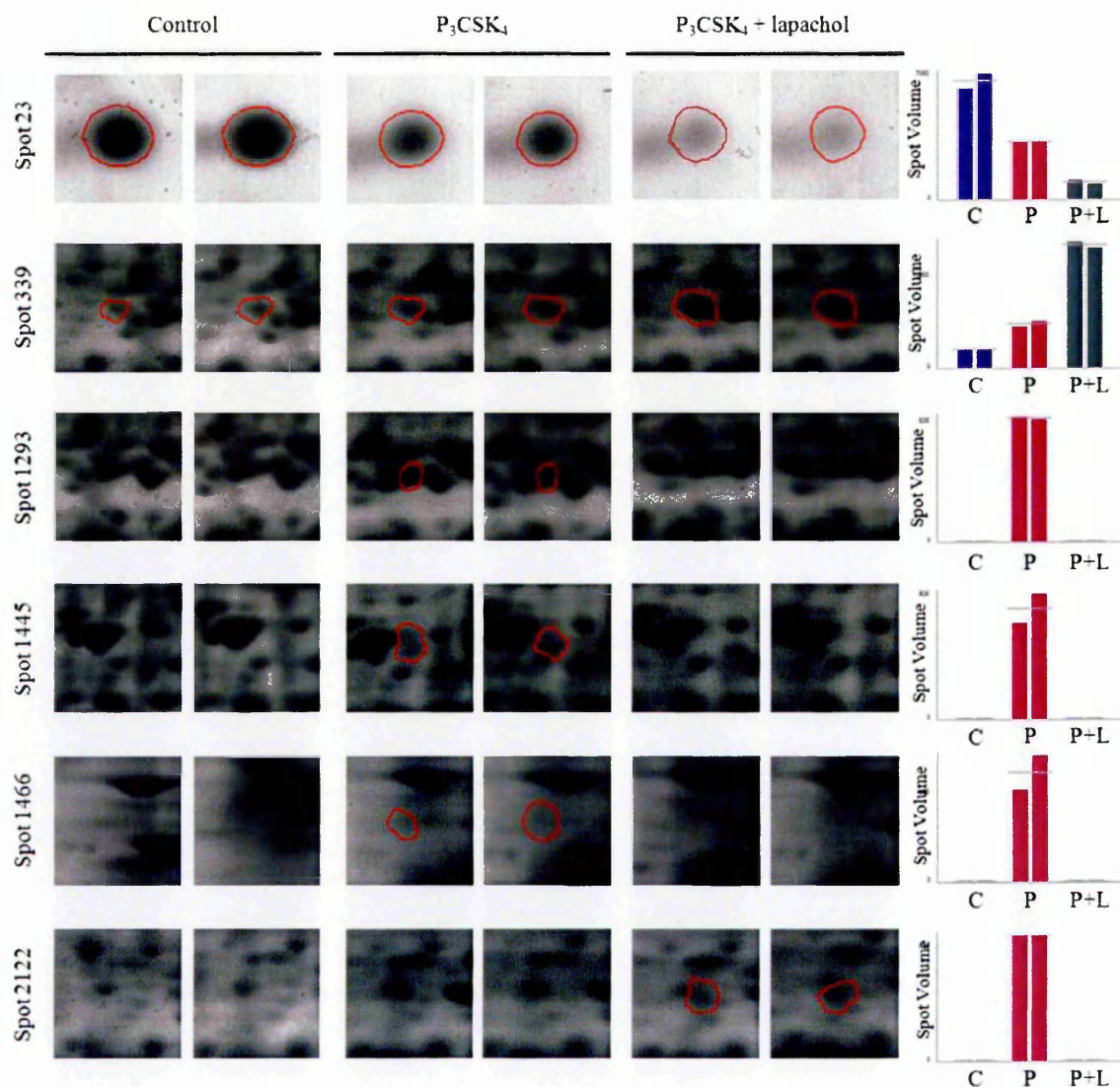
Cells were cultured in the absence of additions for 8 days (A); without additions for 4 days and then with lapachol (32 µg/mL) for 4 days (B); in the presence of P₃CSK₄ (300 nM) for eight days (C); with P₃CSK₄ (300 nM) for 8 days and lapachol (32 µg/mL) from day 4 to 8 (D).

2D-PAGE: 1st dimension IEF, 2nd dimension SDS-PAGE (mol. masses of markers in kDa). Staining: colloidal Coomassie. The corresponding images were analysed, yielding 1260-1350 spots per gel.

The spots encircled blue or red correspond to P₃CSK₄-regulated proteins that were affected by lapachol, and were identified (Table 3). The red circles are the result of a technical feature of the gel image analysis program (Ludesi Redfin); they have the same meaning as the blue ones.

Cellular fraction	SpotNr	Protein	Swiss Prot Acc. Nr	Cellular function	Score	Exp MW/pi	Theor MW/pi	Number of peptides detected	Fold change	
									P ₃ CSK ₄ vs. Control	P+L vs. P ₃ CSK ₄
Cytosol	23	Cofilin-1	P23528	Actin binding	95	19.9/7.90	18.3/8.26	6	↓ 2.07	↓ 3.26
Cytosol	339	Glu-6-P-1-dehydrogenase	P11413	Pentose phosphate pathway	204	52.4/6.25	59.1/6.41	19	↑ 2.32	↑ 2.80
Cytosol	1293	Cytosol amino-peptidase	P28838	Proteolysis	234	56.0/6.75	56.1/8.03	7	↑ ∞	↑ ∞
Cytosol	1445	Fascin	Q16658	Actin binding	317	75.8/6.90	54.3/6.81	9	↑ ∞	↑ ∞
Cytosol	1466	Plastin-2	P13796	Actin binding	130	63.0/5.0	70.1/5.2	8	↑ ∞	↑ ∞
Cytosol	2122	Tryptophan-tRNA ligase	P23381	Tryptophan-RNA ligation	112	52.0/6.00	53.0/5.83	4	↑ ∞	↑ ∞
Membrane	32	Grp78 (BIP)	P11021	ER stress	908	64.0/4.90	70.4/5.01	24	↓ 2.53	↑ 2.07
Membrane	274	Proteasome subunit beta type-4	P28070	Proteolysis	127	25.0/5.40	24.3/5.47	4	↑ ∞	↑ ∞
Membrane	327	Cathepsin D	P07339	Proteolysis	149	25.0/5.3	37.8/5.6	10	↓ 2.05	↑ ∞
Membrane	1391	2,4-dienoyl-CoA reductase	Q16698	Fatty acid metabolism	171	38.0/9.90	32.1/8.79	7	↑ 7.19	↓ 2.09
Membrane	1860	Mn-Superoxide dismutase	P04179	Superoxide dismutation	269	20.0/6.50	22.2/6.86	8	↑ ∞	↓ 1.67

Table 4. Proteins differentially expressed upon P₃CSK₄-treatment whose expression levels were affected by lapachol. Differentially expressed proteins due to P₃CSK₄ treatment for 8 days (P₃CSK₄ vs. Control column, values ≥ 1.5-fold), the expression of which changed when lapachol was present during the last 4 days (P+L vs. P₃CSK₄ column, values ≥ 1.5-fold). Protein names and functions are according to UniProt Knowledge base (SwissProt + TrEMBL) (<http://expasy.org/sprot/>). P+L : P₃CSK₄ + lapachol; Exp. : Experimental; Theor. : Theoretical; MW : Molecular weight; pl: Isoelectric point.

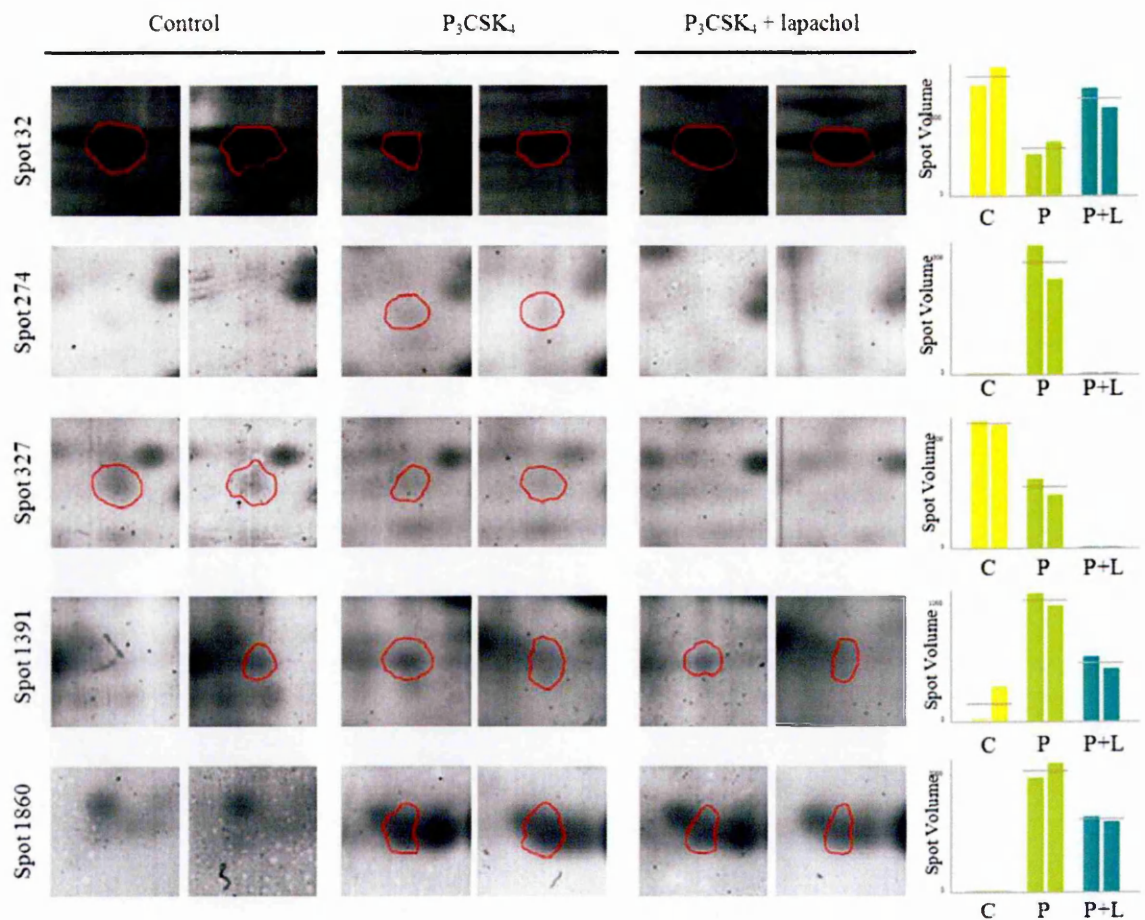


spot 23 : Cofilin-1; spot 339 : G6PD; spot 1293: Amino-peptidase
spot 1445 : Fascin; spot 1466 : Plastin-2; spot 2122 : Tryptophan-tRNA ligase

FIGURE 47. Detail of the alterations induced by lapachol on P₃CSK₄-regulated cytosolic protein expression.

Cells were cultured in the absence of additions for 8 days (**Control**), in the presence of P₃CSK₄ for eight days (**P₃CSK₄**); with IFN- γ for 8 days and additionally lapachol (32 μ g/mL) from day 4 to 8 (**P₃CSK₄ + lapachol**). Cytosolic proteins were analysed by IEF/ SDS-PAGE, in duplicate. Gel images were subjected to quantitative analysis (www.ludesi.com), which includes a normalization step. The figure illustrates the areas of the gels showing proteins (red encircled spots) regulated by P₃CSK₄ activation, the expression of which was affected by lapachol. The bar graphs show the quantification of the spots, in each case.

C: Controls (untreated cells); P: P₃CSK₄; P+L: P₃CSK₄ + lapachol.



spot 32 : Grp78 (BIP); **spot 274** : Proteasome subunit beta type-4;
spot 327: Cathepsin D; **spot 1391** : 2,4-dienoyl-CoA reductase;

spot 1860 : MnSOD

FIGURE 48. Detail of the alterations induced by lapachol on P₃CSK₄-regulated membrane protein expression.

Cells were cultured in the absence of additions for 8 days (**Control**), in the presence of P₃CSK₄ for eight days (**P₃CSK₄**); with IFN- γ for 8 days and additionally lapachol (32 μ g/mL) from day 4 to 8 (**P₃CSK₄ + lapachol**). Membrane proteins were analysed by IEF/ SDS-PAGE, in duplicate. Gel images were subjected to quantitative analysis (www.ludesi.com), which includes a normalization step. The figure illustrates the areas of the gels showing proteins (**red encircled spots**) regulated by P₃CSK₄ activation, the expression of which was affected by lapachol. The bar graphs show the quantification of the spots, in each case.

C: Controls (untreated cells); P: P₃CSK₄; P+L: P₃CSK₄ + lapachol.

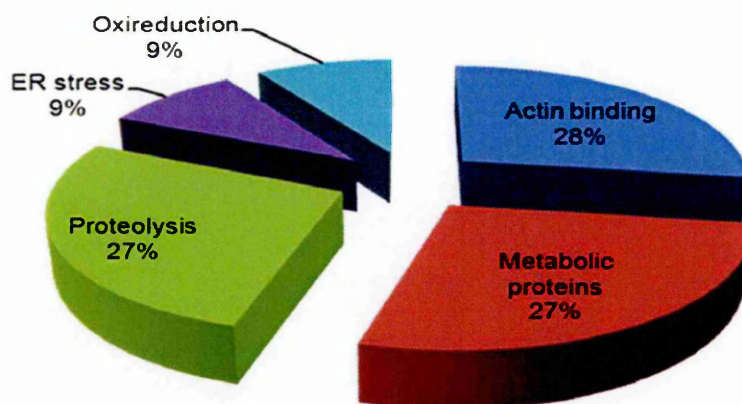


FIGURE 49. Functional classification of the P₃CSK₄-regulated proteins the expression of which was influenced by lapachol.

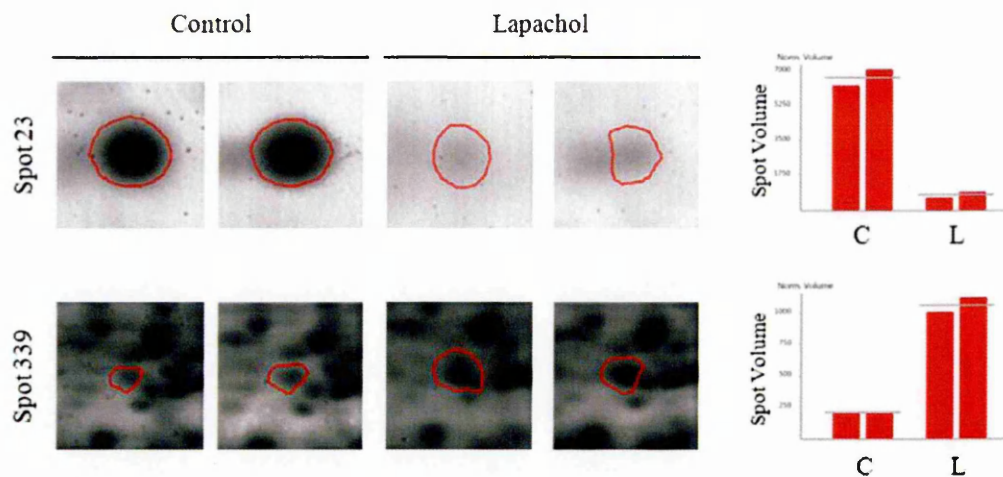
Pie charts representing the distribution of identified proteins according to their biological function. Assignments were made based on information from the Swiss-Prot/TrEMBL protein database (<http://www.expasy.ch>).

3.1. Actin-binding

Cofilin-1 (spot 23) was down-regulated by TLR2 agonism and the combination of TLR2 agonism and lapachol diminished even more the levels of this protein in cytosolic fractions (Table 4, Fig. 47). Fascin (spot 1445) and Plastin-1 (spot 1466) were up-regulated by P₃CSK₄ from undetectable values in resting cell cytosols, and down-regulated back to undetectable amounts by the addition of lapachol (Table 4, Fig. 47). Actin polymerization is fundamental for cellular functions like motility, cell adhesion and phagocytosis. Actin polymerization and depolymerization is regulated by actin-binding proteins (Wear, Schafer et al. 2000). Polymerization occurs at one end of each actin filament, and the control of filamentous actin assembly depends on the regulation of the availability of these ends. Cofilin is an actin-binding protein responsible for the generation of available ends by cleavage of existing F-actins, acting in this way to regulate cell migration (van Rheenen, Condeelis et al. 2009). This was demonstrated by a reduction in cofilin expression by

introduction of cofilin siRNA in neutrophils, which abrogated IL-18-induced cell migration (Hirayama, Adachi et al. 2007). The decrease in cofilin expression caused by TLR2 agonism and accentuated by addition of lapachol could compromise to some degree the migration capacity of macrophages. Migration is essential for phagocytes to reach infection/inflammation sites from blood vessels under the effect of chemotactic stimuli. Once having reached those sites, phagocyte challenging by microbial PAMPs occurs and this leads to phagocyte activation and the mounting of the fight against the invading microorganisms. At this point phagocytes do not need to migrate any longer. In agreement with this scenario of reduced mobility and enhanced killing capacity, reduction in cofilin expression by antisense oligonucleotides has been reported to enhance superoxide production in mouse macrophages (Adachi, Takeuchi et al. 2002). In the present work, the observed increases in the expression of the NADPH-oxidase components gp91^{phox} and/or p47^{phox} by TLR2 agonism and/or *M. avium* infection of macrophages (Fig. 18) coincide with the reduction in cofilin detected by proteomics. Lapachol on its own resulted in decreased cofilin levels similar to those after combined P₃CSK₄/lapachol treatment (Fig. 50). Therefore, lapachol could be inducing an impairment in migration.

Fascin and L-plastin are actin-binding proteins with the capacity to bundle F-actin filaments (Arpin, Friederich et al. 1994; Adams 2004). Their dramatic up-regulation after treatment with P₃CSK₄ (Table 4, Fig. 47) could be linked with the cofilin down-regulation mentioned above, which would increase the cytosolic F-actin content (Adachi, Takeuchi et al. 2002). Since lapachol abolished the P₃CSK₄-induced increase, actin filament bundling would be impaired, with possible consequences regarding cell adhesion, phagocytosis and/or migration.



spot 23 : Cofilin-1
spot 339 : G6PD

FIGURE 50. Detail of two of the alterations induced by lapachol on the cytosolic protein expression of resting cells. Cells were cultured in the absence of additions for 8 days (**Control**), or in the presence of lapachol (32 μ g/mL) from day 4 to 8 (**lapachol**). Cytosolic proteins were analysed by IEF/ SDS-PAGE, in duplicate. Gel images were subjected to quantitative analysis (www.ludesi.com), which includes a normalization step. The figure illustrates the areas of the gels showing proteins (red encircled spots) regulated by lapachol. The bar graphs show the quantification of the spots, in each case. C: Controls (untreated cells); L: lapachol.

3.2. Metabolism

Lapachol affected the levels of some P₃CSK₄-induced enzymes involved in metabolic reactions. Cytosolic tryptophan-tRNA ligase (TrpRS, spot 2122) was strongly up-regulated by P₃CSK₄ treatment, with lapachol reverting completely this effect (Table 4, Fig. 47). TrpRS is an aminoacyl-tRNA synthetase that activates tryptophan by catalyzing the aminoacylation of 3'-terminal adenosine residues in its cognate tRNAs (Kisselev 1993). The effects of this protein are not restricted to the initial steps in protein biosynthesis. After proteolytic cleavage by extracellular proteases such as plasmin or by alternative splicing, small TrpRS are generated that exert an anti-angiogenesis effect on tumor endothelial cells (Kapoor, Zhou et al. 2008; Zhou, Kiosses et al. 2008). IFN- γ increases the expression and activity of TrpRS in amnion cells (Fleckner, Rasmussen et al. 1991) and fibroblasts (Rubin, Anderson et al. 1991), while TGF- β is inhibitory. The up-regulation of TrpRS

levels observed after P₃CSK₄ treatment of THP-1 macrophages can be a cellular response to increase metabolism or could be related to iNOS (NOS2) activity. An unusual TrpRS has been reported to interact with and potentiate the activity of iNOS in prokaryotes (Buddha, Keery et al. 2004), and this might also be the case in eukaryotes. TLR2 agonism resulted in an increase of NOS2 levels (Fig. 20, A), which might be enhanced by TrpRS. This effect would not be operating in TLR2-agonised cells treated with lapachol, since in that case TrpRS levels decrease to undetectable levels (Table 4, Fig. 47).

Glucose-6-P-dehydrogenase (G6PD, spot 339) was up-regulated by TLR2 agonism and the addition of lapachol further enhanced its levels (Table 4, Fig. 47). This is the first enzyme of the pentose phosphate pathway, catalyzes the formation of NADPH and is rate-limiting. It is involved in the redox equilibrium of the cell because NADPH, of which G6PD is an important source, is needed for both the generation of reduced glutathione (GSH) by glutathione reductase and the formation of active catalase tetramers (Pandolfi, Sonati et al. 1995; Salvemini, Franze et al. 1999; Ho, Cheng et al. 2007) that result in the conversion of H₂O₂ into H₂O by glutathione peroxidase and catalase. Treatment of macrophages with P₃CSK₄ resulted in a 2.3-fold increase in G6PD which in association with lapachol became 6.4-fold (Table 4, Fig. 47). Lapachol on its own induced a 5-fold increase (Fig. 50). This effect could help protect macrophages from reactive oxygen species. The lapachol-induced enhancement of G6PD levels could be related to an activation of NADPH-dependent cytochrome P450 reductase by the naphthoquinone, which would require additional NADPH (Kumagai 1997).

Dienoyl-CoA-reductase (spot 1391) was strongly up-regulated in the membrane fraction of P₃CSK₄-treated macrophages and the addition of lapachol halved its expression (Table 4, Fig. 48). Fatty acids are degraded by β -oxidation, in mitochondria. The protein 2,4-dienoyl-CoA-reductase participates as an auxiliary enzyme in fatty acid oxidation. It can process saturated and unsaturated molecules by catalyzing the reduction of 2,4-

dienoyl-CoA to enoyl-CoA in a NADPH-dependent manner (Alphey, Yu et al. 2005; Yu, Chu et al. 2005). The increases in 2,4-dienoyl-CoA-reductase levels caused by TLR2 agonism in THP-1 macrophages might be a consequence of a higher fatty acid turnover/energy requirement. The increase in this enzyme is reduced by lapachol, but levels are still higher than in untreated, control cells.

3.3. Proteolysis

Proteolytic enzymes catalyze the degradation of proteins. Aminopeptidase (spot 1293) and proteasome endopeptidase (spot 274) were both up-regulated by P₃CSK₄ and down-regulated on addition of lapachol. Instead, cathepsin D (CatD, spot 327) was down-regulated by P₃CSK₄ and its levels were reduced further by lapachol.

Aminopeptidase and proteasome endopeptidase are involved in the ATP-dependent proteolysis of cytosolic proteins (Kristensen, Johnsen et al. 1994; Herrera-Camacho, Rosas-Murrieta et al. 2007). Both proteins are involved in the generation of peptides for antigen presentation to CD8⁺ cells via MHC I (Harris, Hunte et al. 1992; Kloetzel and Ossendorp 2004; Hammer, Gonzalez et al. 2007). Up-regulation in the expression of proteasome by TLR2 agonism could be the consequence of the capacity of the cell to improve antigen presentation following pathogen recognition, which lapachol opposed.

Cathepsin D (CatD) is an abundant lysosomal cysteyle protease which promotes an initial cleavage of proteins (Minarowska, Gacko et al. 2008). CatD was shown to be dispensable for MHCII-dependent antigen presentation, using splenocytes from Cat D^{-/-} mice (Deussing, Roth et al. 1998). The reduction in CatD levels by P₃CSK₄ and lapachol would therefore be inconsequential regarding MHCII presentation. No interpretation can be provided at this stage about the possible effects of the observed reduction in lysosomal proteolysis by CatD.

3.4. ER stress

Grp78 is a chaperone that orchestrates the unfolded protein response (UPR) in ER, after the accumulation of unfolded or misfolded proteins in this organelle. Its expression is an ER stress indicator, since grp78 is a central regulator of ER homeostasis (Xu, Bailly-Maitre et al. 2005; Li, Ni et al. 2008). P₃CSK₄ treatment down-regulated the expression of grp78 (spot 32, Table 4 and Fig. 48), in agreement with the reduction in grp78 detected by Western blotting (Fig. 17, A). Lapachol restored grp78 levels to those in non-treated cells.

3.5. Redox state

The mitochondrial Mn-superoxide dismutase (MnSOD, spot 1860) is a main enzyme in the control of cell damage by the enhanced oxygen metabolism that results from infection-induced macrophage activation. It catalyses the conversion of O₂⁻ to H₂O₂ and O₂ (Zelko, Mariani et al. 2002). The expression of MnSOD can be increased by cytokines such as TNF- α (Wong 1995) and treatment with lipopolysaccharide (Gadgil, Pabst et al. 2003), preventing excessive oxidative stress. TLR2 agonism by P₃CSK₄ has been reported to result in a 1.43-fold increase in MnSOD in macrophages (Xue, Yun et al. 2008). The present work showed that MnSOD was noticeably up-regulated by TLR2 agonism, and a modest reversion of this effect was induced by lapachol (Table 4, Fig. 48).

The first general observation from the proteomics section of the studies here reported is that treatment of THP-1 macrophages with the naphthoquinone lapachol resulted in some alterations in protein expression levels. In resting cells, the intensity of 1.7% and 4.6% of cytosolic and membrane fraction spots, respectively, were affected by lapachol treatment. In IFN- γ -treated cells the figures were 7.2% and 9.2% and in TLR2-agonised cells they were 14.7% and 15.7% for cytosolic and membrane spots, respectively.

The total number of proteins that could be identified among those up- or down-regulated by lapachol was 10 from IFN- γ -treated cells and 11 from TLR2-agonised cells. These proteins are involved in a broad spectrum of cellular functions (Figs. 46 and 49) and the possible consequences of the lapachol-induced changes here reported are discussed in the corresponding sections of this Chapter 4 (2.1 to 2.5 and 3.1 to 3.5). Considering more relevant those lapachol-induced changes in protein expression levels where the drug reverts a total abrogation or eliminates completely an up-regulation (returning to undetectable levels) generated by treatment with either IFN- γ or P₃CSK₄, the following 7 proteins are selected: SUMO-1 (#914, Fig.42), DnaJ homolog (#586, Fig. 43), fascin (#1445, Fig. 47), plastin-2 (#1466, Fig. 47), tryptophan-tRNA ligase (#2122, Fig. 47), aminopeptidase (#1293, Fig. 47), proteasome β type-4 protease (#274, Fig. 48). The down-regulation of cofilin (#23, Fig. 47) is also interesting because of its extent. The diversity of the protein functions does not allow drawing any clear conclusion about the possible mechanism of action of lapachol. Such analysis is difficult because: **a.** the number of proteins identified is relatively small, and **2.** the lapachol-induced changes observed could be secondary to upstream event/s, or the result of feed-back mechanisms of compensation. Nevertheless, the down-regulation of the actin-binding proteins cofilin, fascin and plastin-2 suggests that lapachol could be influencing cell migration, phagocytosis and/or immunological synapses. Experimentation addressed to clarify this issue would provide more accurate information about possible impairments.

Apart from the proteins mentioned above, the clear enhancement of glucose-6-P-dehydrogenase (#339, Fig. 47) by lapachol seems interesting. This enzyme generates NADPH, therefore measurement of cellular NADPH concentrations as well as the determination of the redox state of the cell (GSH/GSSG ratios) would be appropriate assessments. Another curious finding is the lapachol-induced enhancement of the cytosolic levels of three normally ER-resident proteins: calreticulin (#1152, Fig. 42), protein

disulfide isomerase A3 (#1266, Fig. 42) and HSP90 (#1275, Fig. 42). This can be due to an activation of retrotranslocation-related processes. Retrotranslocation of calreticulin has been reported and shown to involve the removal of the signal peptide (Afshar, Black et al. 2005). Protein disulfide isomerase has also been reported to be present in the cytosol of Hep3B and cancer cells (Turano, Coppari et al. 2002; Na, Park et al. 2007).

GENERAL DISCUSSION

This study reports a dual activity of the substituted hydroxy-naphthoquinone lapachol. Firstly, it showed anti-mycobacterial (*Mycobacterium avium*) activity at both extra-cellular and intra-macrophage level, a previously unknown effect. Secondly, it immunomodulated some resting and TLR2-agonised human macrophage functions in a manner consistent with an enhanced capacity to curb the intra-cellular growth of mycobacteria. Favourable effects on the cell host were an enhancement of IFN γ R1 and MHCII surface expression and a marked inhibition of IL-10 secretion. Besides, important aspects of host cell function such as ER or oxidative stress, capacity to produce oxygen and nitrogen metabolites, and TNF- α secretion were not affected by the naphthoquinone. The influence of lapachol on the expression of proteins modulated by IFN- γ or TLR2 agonism was studied using a proteomic approach. This allowed the identification of proteins affected and led to hypotheses about the possible consequences of the changes observed.

Like other pathogenic mycobacteria, *M. avium* is an intracellular microorganism able to subvert the innate immune response. As a result, it can persist and replicate within macrophages as well as generate granulomas, a hallmark of mycobacterial infections (Hansch, Smith et al. 1996). In the present studies, *M. avium* replication within macrophages was observed to be biphasic (Fig. 14, A, upper curve), showing an initial inhibition followed by a faster replication. This phenomenon was described by Douvas et al (Douvas, May et al. 1992) and attributed to some serum factors able to inhibit transiently the replication of *M. avium* within macrophages.

During this work, the plant-derived compound lapachol was found to be active against *M. avium* growing in liquid culture, with a MIC of 32 μ g/mL (132 μ M). This concentration is smaller than that reported to inhibit the growth of other bacteria (Guiraud, Steiman et al. 1994; Oliveira, Miranda et al. 2001; Riffel, Medina et al. 2002), of the same order as that needed by the naphthoquinone plumbagin to control *M. smegmatis* and *M. tuberculosis*

liquid growth (Jimenez-Arellanes, Meckes et al. 2003), and higher than that of traditional antibiotics such as isoniazid regarding *M. tuberculosis* growth. Lapachol was not apoptotic nor necrotic toward the THP-1 macrophage-like cells used as host cells for intracellular growth. The cytotoxicity of lapachol toward different tumor cell lines has been studied, in relationship with the use of this compound for the treatment of tumours in soft tissues (Rao, McBride et al. 1968). Leukemic cell lines seem to be more sensitive to lapachol than solid tumors, with cytotoxicity being induced by lapachol at concentrations ranging from 16 to 25 μ M (Salustiano, Netto et al. 2009). Teixeira et al (Teixeira, de Almeida et al. 2001), as part of their investigation on the anti-leishmanial activity of lapachol, exposed mouse peritoneal macrophages to different concentration of this naphthoquinone and found it to be cytotoxic from 0.1 mg/mL (413 μ M). The experiments reported here show that lapachol is not toxic to THP-1 macrophages at $\leq 32\mu$ g/mL (132 μ M).

At its MIC, lapachol exerts a bacteriostatic effect, i.e. it prevents the growth of *M. avium* in liquid culture. This means that, physiologically, the immune system would have to take charge of eliminating the pathogen. Intra-macrophage *M. avium* proliferation was halted at 16 μ g/mL (66 μ M), a concentration smaller than the MIC. The better efficacy of lapachol toward intracellular microorganisms could be due to a higher intracellular concentration, helped by its lipophilic nature (Lira, Sester et al. 2008); a higher stability within the phagosomal environment, protected from clearing processes; an intra-macrophage conversion into a more active derivative; and/or a hypothetical inhibition of bacterial NO reductase/s, the scavengers of macrophage-produced NO (Watmough, Field et al. 2009). The anti-mycobacterial activity of lapachol is likely to be caused by interference with the bacterial cytochrome bc system, a known property of naphthoquinones (Kumagai 1997), and/or by induction of bacterial oxidative stress due to the redox-cycling properties of naphthoquinones, as reported for plumbagin and menadione (Guerra-Lopez, Daniels et al. 2007). In any case, the action of naphthoquinones is related to their involvement in redox reactions.

Another possible reason for lapachol to be more efficient toward intra-macrophage *M. avium* could be the effects it has on some macrophage responses. Lapachol did not alter significantly the resting levels and/or the activation-induced (IFN- γ , TLR2 agonism, *M. avium* infection) increases in the expression of the L-arginine metabolism enzymes NOS2 (NO synthesis) or arginase-1, a negative regulator of NO generation (Fig. 20). Thus, NO generation is likely to be unaffected. Moreover, lapachol did not influence the levels and/or activation-induced increases of the oxygen metabolism markers gp91^{phox} (NADPH oxidase catalytic component), p47^{phox} (NADPH oxidase regulatory component) (Fig. 18) and MnSOD (oxidative stress control) (Fig. 19); nor those of the ER stress marker grp78 (Fig. 17). These data indicate that important microbicidal and detoxifying mechanisms of macrophages are not affected by the naphthoquinone. Hence, the role of these cells in the fight against microorganisms other than mycobacteria would not be compromised during periods of treatment with lapachol, and their action against mycobacteria could actually be improved. An important finding that could result in better responses to IFN- γ and therefore better anti-microbial effects is the lapachol-induced increase in surface IFN- γ R1 (Fig. 33).

Reactions involving quinones can generate superoxide and promote oxidative stress. The fact that lapachol did not induce MnSOD increases (Fig. 19) suggests that this naphthoquinone in particular does not induce harmful oxidative stress at mitochondrial level.

The *M. avium*-induced increase in the secretion of TNF- α and IL-10 by macrophages observed during the present studies (Fig. 32) agrees with published work (Reiling, Blumenthal et al. 2001). The secretion of these cytokines is signaled by MAP kinases (Chanteux, Guisset et al. 2007), which are also related to mechanisms of mycobacterial survival. In fact, intracellular growth of highly replicative *M. avium* is associated to lower levels of MAP kinase phosphorylation and less secretion of TNF- α and IL-10 (Blumenthal, Ehlers et al. 2002). IL-10 is a key anti-inflammatory factor that mediates the inhibition of

proinflammatory cytokine secretion and favours the intracellular growth of microorganisms (Fiorentino, Zlotnik et al. 1991; Bermudez and Champs 1993; Vieth, Will et al. 1994; Via, Fratti et al. 1998; Meghari, Bechah et al. 2008). The absence of IL-10 in knockout mice has been reported to increase the acidification (maturation) of the mycobacterial phagosome (Via, Fratti et al. 1998), which is essential to prevent intracellular bacterial growth. This is due to the fact that IFN- γ promotes phagosomal acidification, and IL-10 inhibits the expression of IFN- γ by T cells (Schaible, Sturgill-Koszycki et al. 1998; Othieno, Hirsch et al. 1999). The *M. avium*-induced secretion of IL-10 elicited by the host macrophages was inhibited by lapachol (Fig. 32), and this inhibition is likely to hamper the intracellular proliferation of mycobacteria by enhancing the growth control capacity of the host cells. The fact that the intra-macrophage growth of *M. avium* was halted by lapachol at 16 $\mu\text{g/mL}$ (66 μM) (Fig. 14, A) as opposed to 32 $\mu\text{g/mL}$ (132 μM) for *M. avium* growing in liquid broth may be related to the lapachol-induced decrease in IL-10 secretion. A smaller concentration of lapachol would be sufficient to stop intracellular growth when the host cell is more efficient, i.e. in the presence of lower levels of IL-10.

The fact that lapachol did not affect the TLR2-induced increase in TNF- α secretion (Fig. 32) nor the corresponding increase in MnSOD (Visner, Dougall et al. 1990) (Fig. 19) is important because TNF- α is necessary to clear mycobacterial and other infections by enhancing oxidative and nitrosative reactions (Roach, Bean et al. 2002), and MnSOD is a necessary superoxide scavenger at times of cell activation/infection. The role of TNF- α is illustrated by the increased susceptibility of animals lacking the TNF gene or where TNF has been neutralized with anti-TNF antibody to granulomatous pathogens such as *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Histoplasma capsulatum* and to attenuated organisms such as *M. bovis* BCG (Kindler, Sappino et al. 1989; Allendoerfer and Deepe 1998; Kaneko, Yamada et al. 1999). The partial inhibition of *M. avium*-induced

TNF- α secretion by lapachol (Fig. 32) is probably due to its causing *M. avium* growth arrest, which would result in decreased macrophage stimulation at phagosomal level compared with that of cells containing a higher number of bacteria because these are actively proliferating.

The results here presented are the first report about the immunomodulatory effects of the naphthoquinone lapachol on stimulated macrophages, and allow a first analysis of its action in infection/inflammation terms. Partially similar immunopharmacological studies have been published on the effect of the natural compounds ceftiofur and albaconol on LPS-stimulated macrophages and of the lapachol analog plumbagin on concanavalin (ConA)-stimulated lymphocytes. Ceftiotur is a cephalosporin antibiotic of veterinary use that down-regulates the LPS-induced secretion of TNF- α , IL-1 β and IL-6 by blocking NF- κ B and MAPKs signaling, but does not affect IL-10. Therefore, it shows anti-inflammatory effects combined with antimicrobial properties (Ci, Song et al. 2008). Albaconol is a prenylated resorcinol isolated from a mushroom that down-regulates LPS-induced TNF- α , IL-6, IL-1 β and NO production; inhibits NF- κ B activation and enhances suppressor of cytokine signalling-1 (SOCS-1) in RAW264.7 cells. This suggests that albaconol is a potential immunosuppressive and anti-inflammatory drug (Liu, Shu et al. 2008; Liu, Shu et al. 2008). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a compound of plant origin and a lapachol analog that possesses a potent anti-tumour activity. It has also been shown to augment the bactericidal activity of macrophages at low concentrations (Abdul and Ramchender 1995). It inhibits ConA-driven mouse T cell proliferation and the secretion of IL-2, IL-4, IL-6 and IFN- γ , as well as NF- κ B activation (Checker, Sharma et al. 2009). Lapachol has been described as an anti-inflammatory compound on the basis of an absence of paw edema in mice after treatment with the drug (de Almeida, da Silva Filho et al. 1990; Lira, Sester et al. 2008). In contrast with the effects of ceftiofur and albanocol on macrophages and those of plumbagin on lymphocytes, lapachol did not inhibit the

TLR2 agonism-induced secretion of TNF- α and IL-1 β by THP1 macrophages (Fig. 32). This is an indication of a different mechanism of action, which might not involve an inhibition of NK- κ B activation.

Quantitative proteomics is a direct way of looking at cell perturbations due to diseases and drug treatments. Proteomic studies concerning the effect of drugs have been mostly performed with cells in culture and are concerned with anti-cancer agents in the majority of cases. No reports about the effect of anti-microbial compounds at cell proteomics level have been published to date. This type of approach can provide useful information such as the individualization of drug activity biomarkers and cellular pathways involved in the mechanism of action of the drug. The results here reported indicate a down-regulation of cofilin, fascin and plastin-2 by lapachol (Table 4, Fig. 46) that could compromise cell movement, which is involved in chemotactic migration and particle ingestion. Such effect would in fact be consistent with the lack of pad inflammation in mice treated with lapachol. The up-regulation of calreticulin (CalR), protein disulfide isomerase A3 (PDI) and the heat shock protein HSP90 in the cytosolic fraction of the cell (Table 3, Fig. 41) could indicate an increased, selective retro-translocation from the endoplasmic reticulum where these proteins are abundant. Retrotranslocation to the cytosol is the means by which misfolded or defectively glycosylated proteins are normally directed for proteosomal degradation. However, these three proteins (CalR, PDI, HSP90) were found intact as to molecular mass, therefore their retrotranslocation might have a yet unknown purpose. The important lapachol-induced enhancement of cytosolic glucose-6-P-dehydrogenase (G6PD) levels observed (Table 4, Fig. 46) could result in an augmented NADPH availability. In this respect, determining the influence of lapachol on the redox state of the host cells would be convenient. An increase in cytoplasmic NADPH could in turn favour the metabolization of the naphthoquinone by NADPH-dependent cytochrome P450 reductase

(Bachur, Gordon et al. 1979; Komiyama, Oki et al. 1979), as well as increasing the potential of the host cell to produce oxygen and nitrogen metabolites.

The *M. avium* growth-arresting activity of lapachol combined with its beneficial immunomodulating effects makes this naphthoquinone a good candidate for future use in *M. avium* infections. Diseases caused by the non-tuberculous pathogen *M. avium* are increasing worldwide (Suharti, Heering et al. 2004). Studies using other mycobacterial species, *M. tuberculosis* in the first place, and strains of different virulence, will define the therapeutical potential of lapachol as an anti-mycobacterial agent in general. It would also be useful to perform *in vitro* studies about the effects of lapachol on macrophage migration and antigen presentation. Finally, *in vivo* studies will be necessary.

Research perspectives based on the results here reported include the study of the antimycobacterial activity of lapachol in hypoxic conditions. Bacilli become dormant under hypoxia, and their low rate metabolism makes them insensitive to the traditionally used antibiotics since these target metabolic pathways that have become inactive. Only metronidazole has been described as active against dormant mycobacteria (Wayne and Sramek 1994). A natural extension of the present studies will be to work with compounds structurally related to lapachol, i.e. 1,4-naphthoquinone derivatives, with the aim of selecting those with similar activities and requiring lower doses. In this respect, several substituted naphthoquinones have been synthesized and tested in several laboratories in relationship with antiplasmodial, antitumoral, antimicrobial, antiprotozoan and insecticide activities (Oliveira, Lemos et al. 2002; Riffel, Medina et al. 2002; Perez-Sacau, Estevez-Braun et al. 2005; Eyong, Kumar et al. 2008). The final objective is to find new, efficient and immuno-pharmacologically convenient anti-mycobacterial compounds to be used in the fight against tubercular diseases (Guerra-Lopez, Daniels et al. 2007).

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